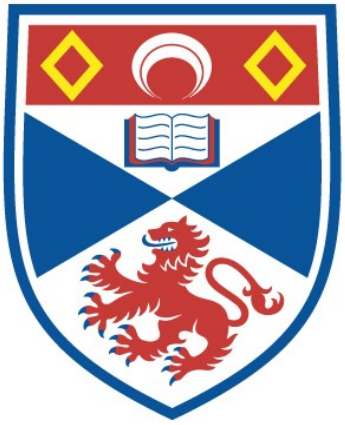
MOLECULAR STUDIES OF POTATO LEAFROLL LUTEOVIRUS MULTIPLICATION

Jane S. Miller

A Thesis Submitted for the Degree of Ph.D.
at the
University of St Andrews



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MOLECULAR STUDIES OF POTATO LEAFROLL LUTEOVIRUS MULTIPLICATION

By

JANE S. MILLER

A thesis presented for the degree of

Doctor of Philosophy

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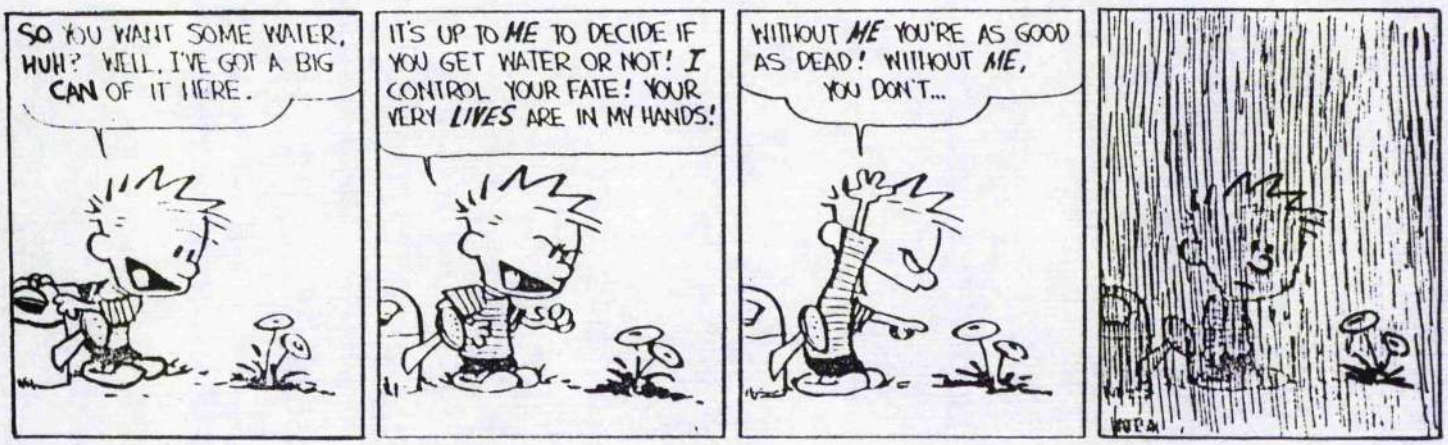
SO YOU WANT SOME WATER, HUH? WELL, I'VE GOT A BIG CAN OF IT HERE.
IT'S UP TO ME TO DECIDE IF YOU GET WATER OR NOT! I CONTROL YOUR FATE! YOUR VERY LIVES ARE IN MY HANDS!
WITHOUT ME YOU'RE AS GOOD AS DEAD! WITHOUT ME, YOU DON'T...

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DECLARATION

I, Jane Smith Miller, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree of professional qualification.

I was admitted to the Faculty of Science at the University of St. Andrews under Ordinance General No. 12 on 1st October 1989 and as candidate for the degree of Ph.D. on 1st October 1989.

CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

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ABBREVIATIONS

AMV - alfalfa mosaic virus

AbMV - abutilon mosaic virus

ArMV - arabis mosaic virus

BCIP - 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt

BSA - bovine serum albumen

BLRV - bean leafroll virus

BMV - brome mosaic virus

BNYVV - beet necrotic yellow vein virus

BWYV - beet western yellows virus

BYDV - barley yellow dwarf virus

BYVBV - bean yellow vein banding virus

CaMV - cauliflower mosaic virus

CarLV - carrot red leaf virus

CCMV - cowpea chlorotic mottle virus

CMoV - carrot mottle virus

CPMV - cowpea mosaic virus

dATP - deoxy adenosine triphosphate

d-aza GTP - deoxy-aza guanosine triphosphate

dCTP - deoxy cytosine triphosphate

dGTP - deoxy guanosine triphosphate

dNTP - deoxy nucleoside triphosphate

ddATP - dideoxy adenosine triphosphate

ddCTP - dideoxy cytosine triphosphate

ddGTP - dideoxy guanosine triphosphate

ddNTP - dideoxy nucleoside triphosphate

ddTTP - dideoxy thymidine triphosphate

DAS-ELISA - double antibody sandwich enzyme linked immunosorbent assay

DTT - dithiothreitol

EDTA - ethylenediaminetetra-acetic acid

ELISA - enzyme linked immunosorbent assay

EM - electron microscopy

FITC - fluorescein isothiocyanate

GRAV - groundnut rosette assistor virus

GRV - groundnut rosette virus

ISDV - Indonesian soybean dwarf virus

LSMV - lettuce speckles mottle virus

MAb - monoclonal antibody

MCMV - maize chlorotic mottle virus

MoMLV - Moloney murine leukaemia virus

MOPS - 3-[N-Morpholino]propane-sulphonic acid

NBT - p-nitro blue tetrazolium chloride

ORF - open reading frame

PBS - phosphate buffered saline, 0.1 M-NaH_2PO_4, 0.1 M-Na_2HPO_4, 0.85% NaCl, pH 7.0.

PCR - polymerase chain reaction

PEBV - pea early browning virus

PEG - polyethylene glycol

PLO - poly-L-ornithine

PLRV - potato leafroll virus

PPO - 2,5-diphenyloxazole

PVP - polyvinylpyrrolidone

RRSV - raspberry ringspot virus

SbDV - soybean dwarf virus

SBMV - southern bean mosaic virus

SDS - sodium dodecyl sulphate

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SSC - standard saline citrate, 3 M-NaCl. 0.3 M-tri-sodium citrate, pH 7.0.

TBE - 1 M-Tris-HCl, 1 M-boric acid, 0.02 M-EDTA, pH8.3.

TBRV - tomato blackring virus

TBS - tris buffered saline, 0.01 M-Tris-HCl, 0.9% NaCl, pH 7.4.

TCA - trichloroacetic acid

TE - 10 mM-Tris-HCl, 0.2 mM-EDTA, pH 7.0.

TMV - tobacco mosaic virus

TMoV - tobacco mottle virus

TNDV - tobacco necrotic dwarf virus

TRSV - tobacco ringspot virus

TRV - tobacco rattle virus

TTP - thymidine triphosphate

TuYV - turnip yellows virus

TY - tryptone yeast

TYMV - turnip yellow mosaic virus

TYVAV - tobacco yellow vein assistor virus

TYVV - tobacco yellow vein virus

UV - ultra violet

VLP - virus-like particle

VPg - genome linked protein

ABSTRACT

Potato leafroll luteovirus is an aphid-transmissible virus which has isometric particles and is confined to the phloem tissue of infected plants. Its multiplication was investigated by using plant protoplasts as a model system.

In protoplasts, net accumulation of PLRV ceased at approximately 48 hrs post-inoculation. Virus-specific products were detectable 15 hrs or more post-inoculation and remained detectable at approximately 100 hrs post-inoculation.

The amount of PLRV accumulated depended on the conditions in which protoplasts were incubated. Incubation at 25°C rather than 20°C and incubation in the dark for a period rather than continuous light resulted in more PLRV accumulation.

RNA extracted from PLRV-infected protoplasts was identical on northern blots to that extracted from leaf tissue of PLRV-infected Maris Piper potato plants. Northern blots of RNA from other plants, some resistant, some susceptible to PLRV multiplication, were very similar. Resistant plants appeared to contain smaller quantities of subgenomic RNA.

The genes at the 3'-end of the genome are expressed by translation of a subgenomic RNA. This was mapped to position 3376 on the PLRV genome and is therefore 2505 nucleotides long. The untranslated leader sequence of 212 nucleotides contains some putative promoter sequences although not in the same order as described for other viruses.

A sequence of 8 nucleotides at the 5'-end of the genomic RNA was found to be repeated at the 5'-end of subgenomic RNA. The complement of this sequence may form part of an internal initiation site for the viral replicase complex in the minus strand RNA. The possibility of the untranslated leader sequence containing several promoters for both subgenomic RNA synthesis and ORF expression is discussed.

Protoplast lysates contained a component that sedimented nearer the top of a sucrose gradient than virus particles. This contained subgenomic RNA and was detectable by ELISA but not by electron microscopy. It was not present in extracts of PLRV-infected plant tissue or in preparations of purified virus particles and may therefore be an unstable structure possibly - playing a role in particle assembly.

1. INTRODUCTION

1.1 INTRODUCTION

Potato leafroll is a disease of potatoes which has major economic importance throughout the world. It is caused by potato leafroll virus (PLRV), which induces symptoms such as leaf yellowing and rolling and is a definitive member of the luteovirus group.

The disease was first reported in 1906 (Appel, 1906) but it was not until 1965 that Peters identified PLRV as the causal agent and purified virus particles from the aphid vector, *Myzus persicae* (Sulz.) (Peters, 1965).

Like the other members of the luteovirus group, PLRV is restricted to the phloem tissue of its host plant (Jensen, 1969; Kojima *et al.*, 1969) and is transmitted by aphids in a persistent manner but is not mechanically transmissible by inoculation with infected sap (Casper, 1988).

Until fairly recently, purification of the virus from infected leaf material was difficult, partly due to the low virus concentrations in plant sap. In 1979, however, Takanami and Kubo (1979) introduced a purification technique which involved the enzyme-assisted maceration of leaf tissue and resulted in much larger yields of virus per kilogram of tissue used.

PLRV has been shown to be capable of infecting and multiplying in protoplasts isolated from plant tissue and this property has been used to gain

information about infection and multiplication processes (Barker and Harrison, 1982).

1.2 THE LUTEOVIRUS GROUP

Group cryptogram [R/1:2/28:S/S:S/Ve/Ap]

Evidence dating from the 18th century suggests that members of the luteovirus group caused various yellowing symptoms found in different crop plants at the time (Duffus, 1972). The word luteo- is derived from the Latin word "luteus" meaning yellow.

The group itself was recognised as a plant virus group in 1975 by the International Committee on Taxonomy of Viruses (Shepherd *et al.*, 1976) and initially consisted of only 3 definitive members; barley yellow dwarf virus (BYDV), the type member of the group, beet western yellows virus (BWYV) and PLRV. The use of serological techniques has established relationships between these existing members and other potential group members. There are currently 10 definitive members (Randles, 1991) and there are several other probable and possible members (Table 1.1). Some of these viruses may, however, be synonyms for already recognised luteoviruses and the number of possible members will probably decrease as isolates are further characterised (Waterhouse *et al.*, 1988).

The type member, BYDV, is currently split into two groups on the

TABLE 1.1: Definitive, probable and possible members of the luteovirus group

(Synonyms are in italics).

from Waterhouse *et al.*, 1988,

Randles, 1991.

DEFINITIVE MEMBERS OF THE LUTEOVIRUS GROUP

Barley yellow dwarf virus subgroup 1	MAV
	PAV
	SGV
Barley yellow dwarf virus subgroup 2	RPV
	RMV
	RGV (rice giallume)

Bean leafroll virus
- *Legume yellows virus*
- *Michigan alfalfa virus*
- *Pea leafroll virus*

Beet western yellows virus
- *Beet mild yellowing virus*
- *Malva yellows virus*
- *Turnip yellows virus*

Carrot red leaf virus
Groundnut assistor virus
Indonesian soybean dwarf virus
Potato leafroll virus
- *Solanum yellows virus*
- *Tomato yellow top virus*

Soybean dwarf virus
- *Subterranean clover red leaf virus*
- *Strawberry mild yellow edge virus*

Tobacco necrotic dwarf virus

PROBABLE AND POSSIBLE MEMBERS OF THE LUTEOVIRUS GROUP

Beet yellow net virus
Celery yellow spot virus
Cotton anthocyanosis virus
Filaree red leaf virus
Milk vetch dwarf virus
Millet red leaf virus
Physalis mild chlorosis virus
Physalis vein blotch virus
Raspberry leaf curl virus
Tobacco vein distorting virus
Tobacco yellow net virus
Tobacco yellow vein assistor virus

basis of differences noted in vector specificity by Rochow (1969). Subgroup 1 contains BYDV isolates which are transmitted by vectors i) *Sitobion avenae* and *Rhopalosiphum padi*, ii) *S. avenae* and iii) *Schizaphis graminum*. These are denoted isolates PAV, MAV and SGV respectively. Subgroup 2 contains isolates RPV and RMV which are transmitted by vectors *R. padi* and *R. maidis* respectively. This evidence has been supported by serological studies (Rochow and Carmichael, 1979), nucleic acid hybridisation studies (Waterhouse *et al.*, 1986) and cytopathological studies (Gill and Chong, 1979). Subsequent observations have shown that most of the other group members also fit into one of these two subgroups (Duffus, 1977; Waterhouse *et al.*, 1988; Martin and D'Arcy, 1990).

1.2.1 DIVERSITY AND ECONOMIC IMPORTANCE OF LUTEOVIRUSES

Luteoviruses are found throughout the world in different climatic regions, infecting many diverse species of plant. Some, such as BYDV, BWYV, carrot red leaf virus (CarLV) and PLRV are widespread, but others, such as tobacco necrotic dwarf virus (TNDV), are restricted to one geographical location (Waterhouse *et al.*, 1988). All the definitive members of the group have natural host ranges mainly restricted to one plant family, with the exception of BWYV which infects many species of several different families (Waterhouse *et al.*, 1988), (Table 1.2).

Heavy economic loss is caused by the infection of crop plants by luteoviruses, BYDV having caused a loss of barley and oats estimated to be worth $35 915 000 in the USA in 1960 (Duffus, 1977). BWYV caused 100% yield loss of infected lettuce plants in England in 1974 (Watts, 1975) and PLRV caused 55% yield loss of infected potatoes in Canada in crops during several years (Nelson and Torfason, 1974). Eradication of these viruses can be difficult due to various weeds and grasses being natural hosts and aphid vectors capable of transmitting the viruses from one plant species to another may also be present.

Different luteoviruses cause diseases of varying severity. For example, most isolates of BYDV and BWYV cause symptoms which are so mild that they are often attributed to non-viral factors, whereas bean leafroll virus (BLRV) and TNDV cause symptoms and stunting so severe that the infected crop can be rendered completely worthless (Waterhouse, 1981).

However, the economic importance of the virus does not depend merely on symptoms expressed in its host. The host range, distribution and prevalence of the virus must also be taken into account. Therefore, although TNDV can cause the almost complete loss of an infected crop, it is restricted to certain areas of Japan only, whereas BYDV, although producing mild symptoms, is ubiquitous and annually causes major, worldwide crop losses.

TABLE 1.2: Hosts and vectors of definitive members of the luteovirus group

MEMBER	MAIN HOST SPECIES	VECTOR SPECIES
BYDV-MAV	Gramineae	*Sitobion avenae*
BYDV-PAV	Gramineae	*S. avenae*
		Rhopalosiphum padi
BYDV-SGV	Gramineae	*Schizaphis graminum*
BYDV-RPV	Gramineae	*R. padi*
BYDV-RMV	Gramineae	*R. maidis*
BLRV	Leguminosae	*Acyrthosiphum pisum*
BWYV	Amaranthaceae	*Myzus persicae*
	Chenopodiaceae	
	Compositae	
	Cruciferae	
	Leguminosae	
	Solanaceae	
CarLV	Umbelliferae	*Cavariella aegopodii*
GRAV	Leguminosae	*Aphis craccivora*
ISDV	Leguminosae	*Aphis glycines*
PLRV	Amaranthaceae	*M. persicae*
	Solanaceae	
SbDV	Leguminosae	*Aulacorthum solani*
TNDV	Solanaceae	*M. persicae*

1.2.2 LUTEOVIRUS TRANSMISSION

Luteoviruses cannot be transmitted by mechanical inoculation with infected sap, and they are thought not to be seed transmissible (Casper, 1988). Instead they are transmitted by aphids in the persistent manner (Table 1.2). Transmission of this kind involves the post-acquisitional circulation of the virus through the gut and into the haemolymph of the aphid (Gildow, 1987). The virus is passed back to the mouth and salivary duct via the accessory salivary glands. At this point the virus is free to be expelled during feeding into plant phloem cells along with secretion products of the salivary gland (Gildow, 1987). The virus can persist in the vector for over 50 days and is retained after moulting (Duffus, 1972).

The efficiency of transmission is increased with longer acquisition and inoculation access times. The minimum access times are 5-240 minutes (Duffus, 1972).

Acquisition is followed by a latent period of 12-24 hours (Duffus, 1972) during which time the virus is probably being circulated through the aphid's body. These times are subject to variation depending on such influences as the efficiency of the vector, the virus concentration in the host plant, the strain of virus, the temperature and other environmental factors (Damsteegt and Hewings, 1987).

There is a high degree of vector specificity involved in the transmission of most luteoviruses; most are transmitted by one vector species only (Waterhouse, 1981; Gildow, 1987). It has been proposed that this

specificity is determined by the salivary glands of the aphid, in particular the basal lamina of the accessory salivary gland (Gildow and Rochow, 1980a; Gildow, 1987). It was also proposed that vector-specific transmission of luteoviruses is based on interactions between the virus capsid protein and a component of the accessory gland cell membrane (Gildow and Rochow, 1980b; Gildow, 1987). This may take the form of membrane receptors which recognise specific virus isolates and hence determine eventual transmission of the virus.

No evidence for virus replication in the vector has been found (Eskandari *et al.*, 1979). This is consistent for all luteoviruses except for conflicting reports on PLRV. Multiplication and antigen accumulation of PLRV have been observed in *M. persicae* by Stegwee and Ponsen (1958) and Weidemann (1982). In contrast, Harrison (1958), and Eskandari *et al.* (1979) reported no PLRV multiplication in the vector.

1.2.3 VIRUS-CELL INTERACTION

It is a characteristic feature of luteoviruses that they are restricted to the phloem tissue of the host plant and that they induce their symptoms by damaging the cells in this tissue.

The type and severity of the symptoms of BYDV infection depend on several factors such as the plant species, variety, age and condition and on environmental factors such as light intensity and temperature. The symptoms

are at their most severe when the plant is infected when young and then grown in conditions of high light intensity and cool temperature. If the plant does not remain in these conditions, it is normal for the symptoms to fade (Rochow, 1970).

Luteoviruses might be limited to the phloem because (Waterhouse, 1981):

1. the necessary conditions for multiplication may only be found in the phloem tissue, and
2. the viruses cannot move laterally from cell to cell, only longitudinally along phloem vessels.

Evidence for the second suggestion is supported by the knowledge that luteoviruses such as BYDV, PLRV and TNDV are able to replicate in tobacco protoplasts inoculated *in vitro* (Barnett *et al.*, 1981; Kubo and Takanami, 1979; Kubo, 1981). Moreover, when TNDV was manually inoculated into epidermal cells of tobacco leaves, it caused an infection but did not spread from the cells which were originally infected (Imaizumi and Kubo, 1980).

Evidence discussed earlier that the luteovirus group in general and BYDV specifically could be split into two subgroups on the basis of vector specificity (Rochow, 1969), serological studies (Rochow and Carmichael, 1979), nucleic acid hybridisation studies (Waterhouse *et al.*, 1986) and genome organisation (Martin *et al.*, 1990) was reinforced by cytopathological studies carried out by Gill and Chong (1979). In this case it was suggested

that the luteoviruses could be divided into two groups depending on events after infection.

In subgroup 1, which contains BYDV isolates MAV, PAV and SGV, filaments and vesicles bound with a single membrane were found in the cytoplasm of infected cells near the plasmodesmata. Filaments were also seen in the nucleus.

A short time after infection, most organelles began to disintegrate and the nucleus was observed to deteriorate quickly. Virus particles did not appear in the nucleus, suggesting that assembly was taking place in the cytoplasm.

The second subgroup, containing BYDV isolates RPV and RMV, and possibly BWYV and PLRV (Esau and Hoefert, 1972; Shepardson *et al.*, 1980), produced vesicles which were bound by two membranes, the second being continuous with the endoplasmic reticulum. In the case of BWYV, fibrillar networks reminiscent of those seen in chloroplasts and mitochondria were present in the vesicles (Esau and Hoefert, 1972). This may suggest that the vesicle contents are associated with nucleic acid. The vesicle was found to associate with the nucleus and BWYV particles were also found at this location suggesting that at least some steps in viral multiplication occur here (Esau and Hoefert, 1972).

Moreover, in this subgroup, the nucleus did not deteriorate as in subgroup 1 and filaments did not appear to be present in the nucleus and were only found in very small quantities in the cytoplasm. Tubular membranous structures were present in larger numbers.

Although these events are found to be similar between different luteoviruses, the cytopathological effects can vary with host species and virus isolate (D'Arcy and de Zoeten, 1979; Gill and Chong, 1981).

1.2.4 LUTEOVIRUS CONTROL

Although luteoviruses are transmitted by aphids, some virus spread is initiated by the planting of infected material, e.g. PLRV-infected tubers (Waterhouse *et al.*, 1988).

Several methods of control are employed to prevent this and aphid transmission (Barker, 1988; Casper 1988). These methods comprise the following:

1: The supply of virus-free stock and the removal of virus source plants. Using reliable diagnostic systems such as enzyme-linked immunosorbent assay (ELISA, Clark and Adams, 1977; Tamada and Harrison, 1980), virus-free stock can be identified and, in some cases, virus-infected stock can be treated to eradicate the virus. Kassanis (1950) developed a method to free potato tubers from PLRV infection. This involved the incubation of the tubers at 37°C in air which appeared to inactivate the virus.

Luteoviruses can spread into susceptible, young, annual crops from infected plants remaining from the previous year's crop, into other crops in adjacent fields and into alternative hosts such as weeds. The removal of the weed hosts and roguing of any plants showing symptoms of secondary

infection while the crop is young, prior to the appearance of vectors, can lead to effective control of luteovirus spread (Barker, 1988).

The use of a crop-free period can also help to remove potentially-infected volunteer plants remaining from a previous year's crop.

2: Crop planting times should be planned to avoid the coincidence of large aphid populations with periods of maximum plant vulnerability, i.e. when the crop is young. Crop location plays an important part in this. For example, *M. persicae*, the aphid vector of PLRV, appears in smaller numbers later in the year in Scotland, owing to the colder winter, than in England and Wales (Woodford *et al.*, 1983). Thus the crop is colonised later, and at a more advanced stage of growth and so is relatively resistant to virus infection by the time the vectors are numerous.

3: Control of the vector population. Insecticides can be used to reduce secondary infection within the crop but most do not prevent primary infection of the crop from another source. Some are more effective than others but there must be a balance kept between the environmental impact of the insecticide and its success.

A non-chemical device has been introduced which consists of the baiting of aphids to sticky yellow polyethylene sheets (Marco, 1981); this has been shown to reduce the aphid population by 70% and is even more effective when used in conjunction with netting.

4: Planting of resistant or tolerant cultivars. There are two main factors involved in this aspect:

i: The plants have greater resistance to infection

ii: The lower concentration of virus particles in leaf tissue makes infected plants less potent sources of inoculum for aphids.

Some varieties of each host plant are naturally more resistant to virus infection than others but the cultivars which show resistance often have a particular trait which makes them unpopular with the farmer or consumer. Breeding programmes have been set up to incorporate resistance into commercially- acceptable new cultivars but these take many years to produce. In dicotyledonous plants, such as tobacco and potato, resistance to certain viruses has been achieved by the transformation of the plant using an *Agrobacterium* vector containing a DNA copy of the coat protein gene of a particular virus (Beachy *et al.*, 1990). Resistance has already been achieved for several viruses, e.g. TMV, AMV and TRV (Abel *et al.*, 1986; van Dun *et al.*, 1987; van Dun and Bol, 1988) and Barker *et al.* (1992) produced plants resistant to PLRV by transformation of this sort.

1.2.5 LUTEOVIRUS PARTICLE PROPERTIES

i. VIRUS PARTICLES

Luteovirus particles are isometric in shape and have diameters ranging from 23-30 nm. Table 1.3a summarises the published properties of luteoviruses basically as in Waterhouse *et al.* (1988), with some recent additions. Synonyms have not been included.

TABLE 1.3a: Luteovirus particle properties

T: Measurment of particles seen in thin sections, *: density as estimated in $CsSO_4$.

References: 1: Rochow, 1970; 2: Brakke & Rochow, 1974; 3: Scalla & Rochow, 1977; 4: Hammond *et al.*, 1983; 5: Paliwal, 1978; 6: Ashby, 1984; 7: Duffus, 1972; 8: Veidt *et al.*, 1988; 9: Hewings & D'Arcy, 1986; 10: Waterhouse & Murant, 1982: 11: Rajeshwari & Murant, 1988; 12: Iwaki *et al.*, 1980; 13: Harrison, 1984; 14: Mayo *et al.*, 1989; 15: Tamada & Kojima, 1977; 16: Kubo, 1981.

MEMBER	PARTICLE DIAM. (nm)	GENOME RNA MWt (X 10^6)	PARTICLE PROTEIN MWt (X 10^{-3})	A_{260}/A_{280}	S_{20w}	DENSITY IN CsCl (g/cm^3)
BYDV-MAV[1]	26,30,24T	1.85[2]	23.5[3,4]	1.88-1.92	115-118	1.39[5]
BYDV-RPV[1]	26,30,24T	1.85-2.0[3,4]	24.4[3,4]	1.71-1.79	115-118	
BLRV[6]	27,23T	2.4	23	1.83		1.32*
BWYV[7]	27,26T	1.9[8]	24[8]	1.65[9]	114[9]	1.42[9]
						1.36*
CarLV[10]	25	1.8	25	1.62	104	1.403
GRAV[11]	29-30	2.1	24	1.86	115	1.34*
ISDV	26,24T[12]					
PLRV[13]	24-25	2.0[14]	26.3	1.78	115-127	1.39
						1.34*
SbDV[15]	25		22	1.96-1.90		
TNDV[16]	25	2.0	25.7	1.8	115	

Thermal inactivation points range from 45°C to 75°C. Generally there is one sedimenting component but a second lighter component has been found in a German isolate of BYDV and a Californian isolate of BWYV (Proll *et al.*, 1985; Hewings and D'Arcy, 1986). These extra components contained virus-like particles with sedimentation coefficients of 55*S* and 62*S* respectively compared with that of 114*S* and 115*S* for the main sedimentable components. The buoyant density of the top component of the BWYV isolate was 1.31 g/ml in CsCl compared with 1.42 g/ml for the main component. Nucleic acid has not been observed to be associated with the virus-like particles of either isolate described.

ii. NUCLEIC ACID AND GENOME ORGANISATION

An initial report which presented evidence describing PLRV to have a genome made up of double-stranded DNA (Sarkar, 1976) has since been discounted. All luteovirus genomes analysed to date consist of single-stranded RNA (Casper, 1988; Waterhouse *et al.*, 1988, Martin *et al.*,1990). The complete nucleotide sequences have been established for BYDV-PAV (Miller *et al.*, 1988), BYDV-RPV (Vincent *et al.*, 1991), BWYV (Veidt *et al.*, 1988) and PLRV (Smith *et al.*, 1988; Mayo *et al.*, 1989; van der Wilk *et al.*, 1989; Keese *et al.*, 1990) and partial sequences have been obtained for BLRV (Prill *et al.*, 1990), SbDV and CarLV (Martin *et al.*, 1990).

Table 1.3b shows these viruses with the relevant reference which details the presence or absence of 1 or more subgenomic RNAs, a virus

TABLE 1.3b: Properties of luteovirus RNA

*: amino acid composition of coat protein available, Waterhouse *et al.*, 1988.

References: 1: Miller *et al.*, 1988; 2: Murphy *et al.*, 1989; 3: Vincent *et al.*, 1991; 4: Prill *et al.*, 1990; 5: Veidt *et al.*, 1988; 6: Falk *et al.*, 1989; 7: Martin *et al.*, 1990; 8: Mayo *et al.*, 1989; 9: van der Wilk *et al.*, 1989; 10: Keese *et al.*, 1990; 11: Tacke *et al.*, 1990; 12: Mayo *et al.*, 1982b.

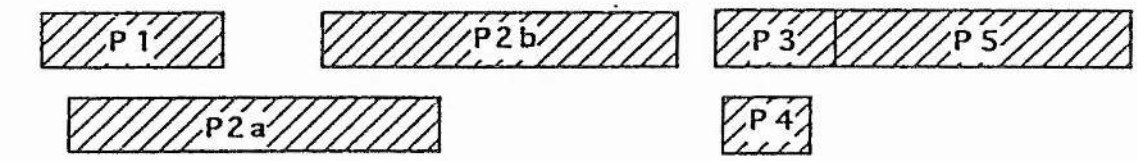
MEMBER	SEQUENCE REF.	No. SUBGENOMIC RNAs	VPg	POLY[A]
BYDV-MAV	1, *	2[1]	+[2]	-[1]
BYDV-RPV	3	1[3]	+[3]	-[3]
BLRV	coat protein only[4]			
BWYV	5	1[6]		-[5]
CarLV	partial sequence only[7]			
PLRV	8,9,10	1[8,11]	+[12]	-[12]
SbDV	partial sequence only[7]*			
TNDV	*			

FIGURE 1.1: Genome organisation of luteoviruses; 2 subgroups.

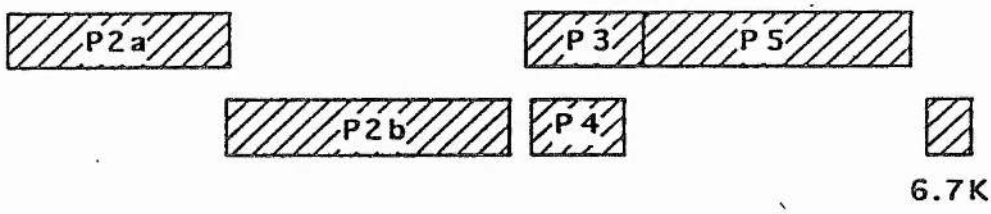
adapted from Martin *et al.*, 1990.

The solid line represents the entire genome of each of the two subgroups of luteoviruses. The hatched boxes represent open reading frames. For PLRV, ORF1 stretches from nucleotides 70-812 and encodes a protein of Mr 28K, ORF 2a stretches from nucleotides 203-2121 and encodes a protein of Mr 70K, ORF 2b stretches from nucleotides 1470-3389 and encodes a protein of Mr 70K, ORF 3 stretches from nucleotides 3588-4213 and encodes a protein of Mr 23K, ORF 4 stretches from nucleotides 3614-4081 and encodes a protein of Mr 17K and ORF 5 stretches from nucleotides 4215-5737 and encodes a protein of Mr 56K (as according to van der Wilk *et al.*, 1989).

PLRV - Scottish



BYDV-PAV



genome-linked protein (VPg) and a poly (A) tail.

There are two main genome organisation maps; one describes the genome organisation of the BYDV-PAV subgroup and the other the BYDV-RPV, PLRV and BWYV subgroup (Martin *et al.*, 1990), (Fig. 1.1).

Each group has 6 open reading frames (ORFs). The two subgroups have similar 3'-terminal organisation whereas the 5'-termini are more varied. Common features of the two genome organisations include

1: overlapping ORFs 1 and 2,

2: ORF 4 entirely encompassed by ORF 3

3: ORF 5 separated from ORF 3 by an amber termination codon.

The distinguishing feature of the PLRV, BWYV group is that it has an extra ORF at the 5'terminus. There is no corresponding ORF in the BYDV-PAV genome.

Functions of the ORFs are as follows (according to Martin *et al.*, 1990), (Table 1.4).

ORFs 1 & 2: these encode the 2 viral amino co-terminal polypeptides of the postulated RNA-dependent RNA polymerase.

ORF 3: coat protein gene.

ORF 4: this ORF is completely encompassed by ORF 3 but is in a different frame. The resulting protein may be a 17K VPg. PLRV has been reported to have a VPg of 7K (Mayo *et al.*, 1982b) and it has been proposed that the ORF may code for a 17K precursor which is subsequently processed to release the VPg (van der Wilk *et al.*, 1989).

ORF 5: this ORF is proposed to be translated by readthrough of the amber

TABLE 1.4: Proteins encoded by predicted open reading frames and the lengths of non-coding regions of 4 luteoviruses.

adapted from Martin *et al.*, 1990.

* from Vincent *et al.*, 1991.

MWt x 10^{-3} OF VIRUS PROTEIN

ORF/NCR	BYDV-PAV	BWYV	PLRV	BYDV-RPV*
1	-	29	28	29
2a	39	66	70	71
2b	60	67	69	72
3	22	22.5	23	22
4	17	19.5	17	17
5	50	51.5	56	43
6	6.7	-	-	-
5'NCR	141 nt	31 nt	71 nt	114 nt
INTERNAL NCR	116 nt	202 nt	196-197 nt	185 nt
3'NCR	568 nt	146 nt	141 nt	102 nt

termination codon of ORF 3(Bahner *et al.*, 1990; Dinesh-Kumar *et al.*, 1992). This results in the synthesis of a protein larger than the expected ORF 5 product. The final product should consist of ORF 3 plus ORF 5 plus any intercistronic region. The 50K (ORF 5) part of the protein is found on the outside of the virus particle and is thought to be involved in the specificity of aphid transmission, acting at the level of transportation of the virions from the haemolymph to the salivary ducts (Waterhouse *et al.*, 1989).

Owing to the constraints imposed on single-stranded RNA viruses, they must employ several strategies in order that their 3'-terminal genes are expressed. One of these strategies is the use of one or more subgenomic RNAs (Morch and Haenni, 1987). The luteoviruses for which the genome organisation and sequence are known have been shown to use this strategy. Those studied so far appear to have one or two subgenomic RNAs (Table 1.3b). This type of RNA is used by viruses to express the genes nearest the 3'-end of the genome, commonly the coat protein gene. RNAs of this nature consequently are of use in the development of coat protein mediated virus resistance as discussed earlier.

In addition to these subgenomic RNAs, a particular isolate, ST9, of BWYV has been shown to have an additional single-stranded RNA (Falk and Duffus, 1984). In infected plants two double-stranded RNAs were present which were not found in plants infected with other isolates. The presence of this additional RNA was associated with severe symptoms. In BYDV, a satellite RNA is present in an RPV isolate (Miller *et al.*, 1991). Its effects on the symptoms produced by the virus are as yet undetermined although when

tested, the isolate did produce severe symptoms in oats. However, this may be due to the genomic RNA itself, the satellite not contributing to the production of symptoms.

The published sequence of the Scottish PLRV isolate (Mayo *et al.*, 1989) contains a 5'-terminal sequence different from those of other isolates (Keese *et al.*, 1990). Recent work has shown this sequence to be that of a minor fraction in the PLRV (Scottish) RNA (Mayo and Jolly, 1991). For convenience, sequence coordinates used here refer to those in the sequence of the Dutch isolate which is one nucleotide shorter at the 5'-end than others (van der Wilk *et al.*, 1989; Keese *et al.*, 1990).

1.2.6 LUTEOVIRUS RELATIONSHIPS WITH OTHER GROUPS

Several luteoviruses are known to assist the aphid transmission of viruses which are not, themselves, aphid transmissible. The luteovirus is known as the "helper virus" and in association with an aphid non-transmissible virus is known as the virus complex (Rochow, 1972).

For example, CarLV is known to be essential in the aphid transmission of carrot mottle virus (CMoV, Watson *et al.*, 1964) and GRAV is needed for the aphid transmission of groundnut rosette virus (GRV, Hull and Adams, 1968, Table 1.5). In luteovirus complexes, the dependent virus can be aphid transmitted only if it is in the same source plant as the helper virus.

TABLE 1.5: Luteoviruses which can act as helper viruses.

from Waterhouse *et al.*, 1988.

References: 1: Rochow, 1975; 2: Rochow, 1982; 3: Rochow, 1970; 4: Creamer & Falk, 1990; 5: Cockbain, 1978; 6: Cockbain, 1986; 7: Falk *et al.*, 1979; 8: Watson *et al.*, 1964; 9: Hull & Adams, 1968; 10: Smith, 1945; 11: Smith, 1946; 12: Adams & Hull, 1972.

HELPER VIRUS	VECTOR	DEPENDENT VIRUS
BYDV-RMV	*Rhopalosiphum maidis*	BYDV-MAV[1]
BYDV-RMV	*R. maidis*	BYDV-RPV[2]
BYDV-RPV	*R. padi*	BYDV-MAV[3]
BYDV-RPV	*R. padi*	BYDV-RMV[2]
BYDV-RPV	*R. padi*	BYDV-SGV[2]
BYDV-PAV	*R. padi*	BYDV-RMV[2]
BYDV-PAV	*R. padi*	BYDV-MAV[2]
BYDV-PAV	*Sitobion avenae*	BYDV-RPV[4]
BLRV	*Acyrthosiphon pisum*	BYVBV[5,6]
BWYV	*Myzus persicae*	LSMV[7]
CarLV	*Cavariella aegopodii*	CMoV[8]
GRAV	*Aphis craccivora*	GRV[9]
TNDV	*M. persicae*	TMoV[10,11]
TYVAV	*M. persicae*	TYVV[12]
TuYV	*M. persicae*	LSMV[7]

The RNA of the dependent virus can be encapsidated in the coat protein of the helper virus and this is thought to explain its transmission by the luteovirus vector (Falk *et al.*, 1979). It has also been shown that the dependent virus can be transmitted by different helper viruses using different vectors and infecting different host plants (Adams and Hull, 1972; Waterhouse and Murant, 1983). This is a very effective manner for some viruses to be transmitted to a wider range of host plants and also introduces the possibility of new, more virulent strains of virus being formed owing to the transfer of genetic material.

1.3 THE USE OF PROTOPLASTS IN PLANT VIRUS RESEARCH

In a similar manner to the development of animal and human tissue culture systems, attempts have been made to develop a plant cell culture system which allows virus infection and multiplication. Initially callus cells were grown in culture but were proven to be difficult to inoculate effici ently with virus particles (Murakishi, 1968).

To overcome this problem, the technique of using suspensions of protoplasts, plant cells which have had their outer cell wall removed, was developed. This has been found to be a more preferable and convenient culture system compared to the others that are available and is now widely used in plant virus research (Table 1.6, Harrison and Mayo, 1983).

TABLE 1.6: Protoplasts infected by plant viruses

1: Okuno *et al.*, 1977; 2: Okuno & Furusawa, 1979; 3: Furusawa & Okuno, 1978; 4: Motoyoshi *et al.*, 1974a; 5: Maekawa *et al.*, 1981; 6: Okuno & Furusawa, 1978; 7: Loesch-Fries & Hall, 1980; 8: Dawson *et al.*, 1978; 9: Watts *et al.*, 1987; 10: Motoyoshi *et al.*, 1973; 11: Morris & de Zoeten, 1990; 12: Kluge *et al.*, 1983; 13: Howell & Hull, 1978; 14: Hussain *et al.*, 1987; 15: Yamaoka *et al.*, 1982; 16: Maule, 1983; 17: Beier & Bruening, 1975; 18: Hibi *et al.*, 1975; 19: Jarvis & Murakishi, 1980; 20: Huber *et al.*, 1977; 21: de Varennes *et al.*, 1984; 22: Beier & Bruening, 1976; 23: Nitta *et al.*, 1988; 24: Lesney & Murakishi, 1981; 25: Fuentes & Leon, 1986; 26: Beier *et al.*, 1981; 27: Fukunaga & Furusawa, 1981; 28: Shanks *et al.*, 1989; 29: Coutts & Wood, 1976b; 30: Koike *et al.*, 1977; 31: Otsuki & Takebe, 1973; 32: Takebe & Otsuki in Takebe, 1975; 33: Maule *et al.*, 1980a; 34: Hirai & Amemiya, 1989; 35: Okada *et al.*, 1988; 36: Linthorst & Kaper, 1984; 37: Osman & Buck, 1987; 38: Paje-Manalo & Lommel, 1989; 39: Pappu & Hiruki, 1988; 40: Kagi *et al.*, 1975; 41: Joersbo & Brunstedt, 1990; 42: Bajet & Goodman, 1981; 43: Townsend *et al.*, 1986; 44: Chiu & Tien, 1982; 45: Loesch-Fries & Hall, 1982; 46: Zheng & Edwards, 1990; 47: Barnett *et al.*, 1981; 48: Dinesh-Kumar *et al.*, 1992; 49: Veidt *et al.*, 1992; 50: Barker & Harrison, 1982; 51: Kubo & Takanami, 1979; 52: Mayo *et al.*, 1982b; 53: Wieringa-Brants *et al.*, 1978; 54: Mayo in Harrison & Mayo, 1983; 55: Mayo *et al.*, 1982a; 56: Barker & Harrison, 1977a; 57: Barker &

Harrison, 1978; 58: Acosta & Mayo, 1990; 59: Barker & Harrison, 1977b; 60: Shalla & Petersen, 1973; 61: Prakash & Foxe, 1985; 62: Ferarra & Tavantzis, 1986; 63: Maule *et al.*, 1980b; 64: Rao & Hiruki, 1978; 65: Bains *et al.*, 1988; 66: Brown & Wood, 1987; 67: Goffinet & Verhoyen, 1979; 68: Barker in Harrison & Mayo, 1983; 69: Dijkstra *et al.*, 1987; 70: Yeh & Chen, 1988; 71: Domier *et al.*, 1989; 72: Riesterer & Adam, 1981; 73: van Beek *et al.*, 1985; 74: Jones & Jackson, 1990; 75: Morris-Krsinich *et al.*, 1979; 76: Rollo & Hull, 1982; 77: Wu *et al.*, 1985; 78: Hanold *et al.*, 1986; 79: Takebe & Otsuki, 1969; 80: Hibi in Takebe, 1975; 81: Otsuki & Takebe in Takebe, 1977; 82: Koike *et al.*, 1976; 83: Coutts & Wood, 1976a; 84: Mayo & Barker, 1983; 85: Motoyoshi & Oshima, 1975; 86: Mertes & Sander in Sander & Mertes, 1984; 87: Langridge *et al.*, 1986; 88: Motoyoshi & Oshima, 1979; 89: Aoki & Takebe, 1969; 90: Sarkar *et al.*, 1974; 91: Fukunaga *et al.*, 1981; 92: Sugimura & Ushiyama, 1975; 93: Kubo *et al.*, 1974; 94: Jones *et al.*, 1990; 95: Russo & Gallitelli, 1985; 96: Renaudin *et al.*, 1975; 97: Muhlbach *et al.*, 1977; 98: Muhlbach & Sanger, 1977; 99: Faustmann *et al.*, 1986; 100: Alblas & Bol, 1977; 101: Motoyoshi *et al.*, 1975; 102: Alblas & Bol, 1978; 103: Samac *et al.*, 1983; 104: Motoyoshi & Hull, 1974.

VIRUS GROUP	VIRUS	PROTOPLAST
Alfamo-	Alfalfa mosaic virus	Cowpea[100,102], tobacco[101], alfalfa[103]
Bromo-	Brome mosaic virus	Barley[1,6,7], Chenopodium hybridum[2], oat[3], wheat[3], radish[3], maize[3], tobacco[4,8], turnip[5], Nicotiana plumbaginifolia[9]
	Cowpea chlorotic mottle virus	Tobacco[8,10], N. plumbaginifolia[9]
Carla-	Potato virus S	Potato[11]
Carmo-	Carnation mottle virus	Dianthus caryophyllus[12]
Caulimo-	Cauliflower mosaic virus	Turnip[13,15], cotton[14], rape[16], mustard[16], Chinese cabbage[16], cabbage[16], white mustard[16], Moricanda arvensis[16]
Como-	Cowpea mosaic virus	Cowpea[17,18,22], soybean,[19], tobacco[20,23], C. quinoa[21]
	Bean pod mottle virus	Soybean[24]
	Bean rugose mosaic virus	French bean[25]
	Cowpea severe mosaic virus	Cowpea[26]
	Radish mosaic virus	Turnip[27]

	Red clover mosaic virus	Cowpea[28]
Cucumo-	Cucumber mosaic virus	Cucumber[29,33], cowpea[30], tobacco[31], Vinca rosea[32], melon[34], rice[35]
	Peanut stunt virus	Cowpea[36]
Diantho-	Red clover necrotic mosaic virus	Cowpea[37], N. clevelandii[38], tobacco[38]
	Sweet clover necrotic mosaic virus	Cowpea[39]
Enamo-	Pea enation mosaic virus	Tobacco[104]
Faba-	Broad bean wilt virus	Broad bean[40]
Furo-	Beet necrotic yellow vein virus	Sugar beet[41]
Gemini-	Bean golden mosaic virus	French bean[42]
	Cassava latent virus	N. plumbaginifolia[43]
Hordei-	Barley stripe mosaic virus	Barley[44,45], oat[46]
Luteo-	Barley yellow dwarf virus	Barley[47], oat[47,48]
	Beet western yellows virus	C. quinoa[49]
	Potato leafroll virus	Potato[50], tobacco[51,52]
	Tobacco necrotic dwarf virus	Tobacco[51]

Marafi-	Maize rayado fino virus	French bean[25]
Necro-	Tobacco necrosis virus	Cowpea[53]
Nepo-	Tobacco ringspot virus	Tobacco[54,55], potato[50], cucumber[33]
	Raspberry ringspot virus	Tobacco[56], N. benthamiana[57], N. clevelandii[58]
	Tomato blackring virus	Tobacco[59,55], potato[50]
Potex-	Potato virus X	Tobacco[60], potato[61], Solanum acaule[62], cucumber[63]
	Clover yellow mosaic virus	Cowpea[64], pea[65,66]
Poty-	Potato virus Y	Tobacco[67,68], potato[68]
	Blackeye cowpea mosaic virus	Cowpea[69]
	Papaya ringspot virus	Cucumis metuliferus[70], C. quinoa[70]
	Tobacco vein mottling virus	Tobacco[71]
Rhabdo-	Potato yellow dwarf virus	N. rustica[72]
	Sonchus yellow net virus	Cowpea[73], N. benthamiana[74], N. edwardsonii[74]
Sobemo-	Southern bean mosaic virus	Soybean[19]
	Turnip rosette virus	Turnip[75,76]
	Velvet tobacco mottle virus	N. clevelandii[77], N. velutina[78]

Tobamo-	Tobacco mosaic virus	Tobacco[79,89,90,8], barley[80], Chinese cabbage[81], cowpea[82], cucumber[83], N. benthamiana[84], petunia[80], tomato[85,88], V. rosea[32,91], soybean[86], carrot[87], potato[50]
	Cucumber green mottle mosiac virus	Tobacco[97]
Tobra-	Tobacco rattle virus	Tobacco[84,93], potato[50], N. benthamiana[57]
Tombus-	Tomato bushy stunt virus	N. benthamiana[94], N. edwardsonii[94]
	Cymbidium ringspot virus	C. quinoa[21], cowpea[95]
Tymo-	Turnip yellow mosaic virus	Chinese cabbage[96], turnip[96]
Viroids	Citrus exocortis viroid	Tomato[97]
	Cucumber pale fruit viroid	Tobacco[98]
	Potato tuber spindle viroid	Tomato[97], potato[99]

1.3.1 THE ISOLATION OF PROTOPLASTS

Protoplasts can be isolated from most parts of a plant and from a wide range of plant species. However, protoplasts isolated from leaves of the tobacco plant, *Nicotiana tabacum*, appear to be the most commonly used (Takebe, 1975). Indeed, the isolation of protoplasts from tobacco leaves (Takebe *et al.*, 1968) and the subsequent infection of these with tobacco mosaic virus (Takebe and Otsuki, 1969) was the foundation for most plant virus studies using protoplasts.

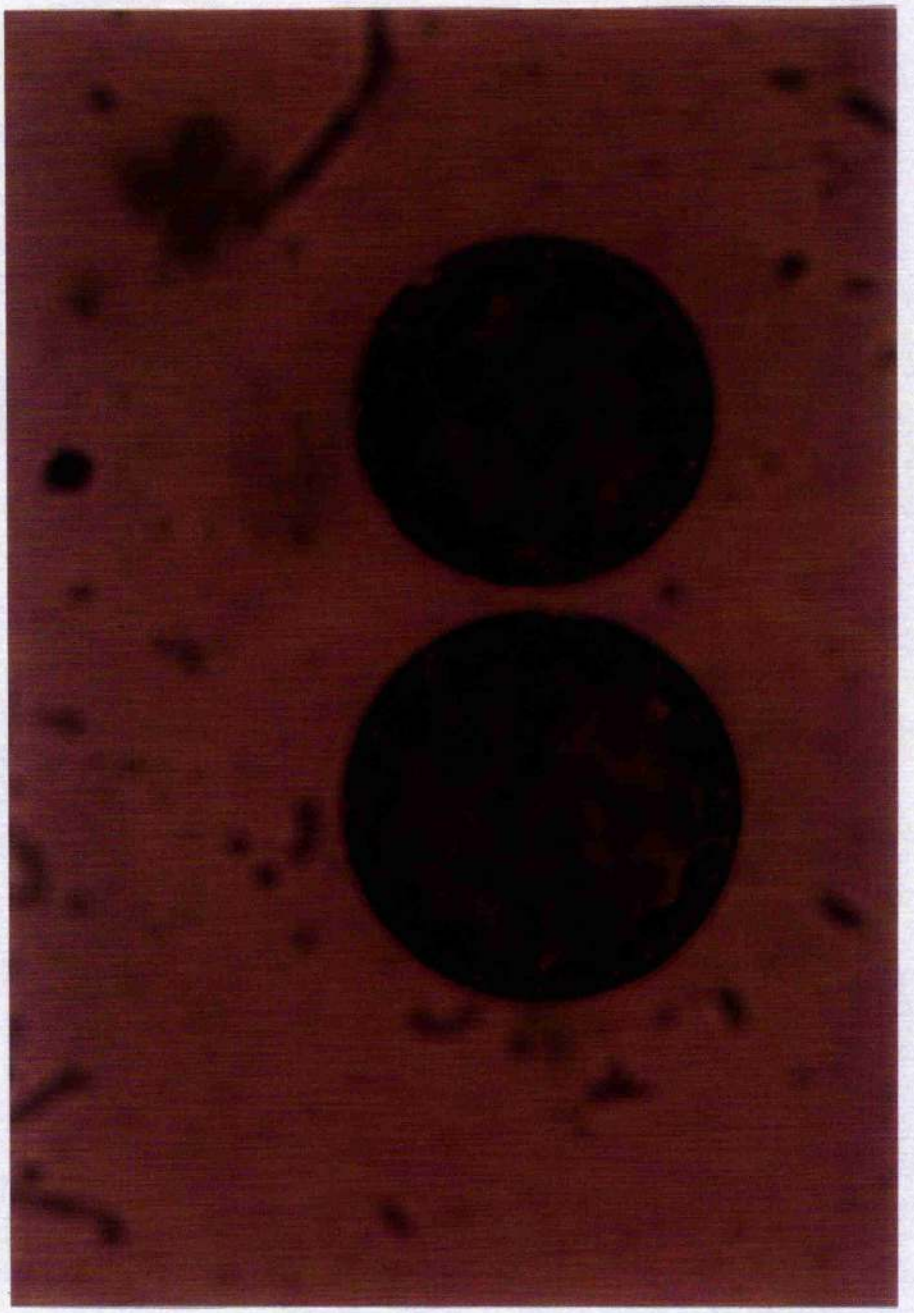
There are two main methods of isolation which are currently in use. The first is the Takebe-Otsuki two step method which involves isolating the desired cells and subsequently removing the outer cell wall (Takebe *et al.*, 1968).

For rapid and efficient isolation, it is necessary to strip the epidermal layer, using forceps, to expose the mesophyll tissue. A pectinolytic enzyme preparation is then used, in the presence of potassium dextran sulphate (which protects the cells from the toxic effects of the enzyme) to separate the cells and provide a population which consists mainly of palisade cells. After separation, cellulase is added to digest the outer cell wall, resulting in the formation of protoplasts (Fig. 1.2).

The second method of isolation is the one step method. This involves treating the exposed mesophyll tissue with a mixture of digesting enzymes to release the protoplasts directly (Otsuki and Takebe, 1969b). This method, although simpler than the two step, yields a more heterogeneous population

FIGURE 1.2: Protoplasts isolated from the plant *Nicotiana tabacum* cv. Xanthi.

Protoplasts were isolated from *N. tabacum* cv. Xanthi plants as described in section 2.3.1. Isolated protoplasts were placed on a glass slide and photographed under a light microscope. Protoplasts were deemed to be in good condition when they appeared to be intact, spherical in shape and very green in colour.



of protoplasts due to the omission of the separation of the palisade cells from the spongy cells.

The final condition of the protoplasts after isolation can depend quite heavily on the age and physiological state of the plants used. Generally, the youngest fully expanded leaves should be used. Kubo *et al.* (1975a) recommended that *Nicotiana tabacum* cv. Xanthi should be grown in a controlled environment in 10 000 lux light for 12 hrs at 25°C alternating with 12 hrs darkness at 20°C. However, seasonal effects such as increased fragility of the protoplasts and poorer levels of infection were still apparent in winter.

The osmotic pressure of all the solutions used in protoplast procedures is very important. All the enzyme solutions are made up in an osmotic stabiliser such as mannitol which prevents lysis of the protoplasts due to a difference in intra- and extra-cellular osmotic pressure.

1.3.2 INOCULATION OF PROTOPLASTS

Protoplasts can be inoculated using either virus particles (Takebe and Otsuki, 1969) or viral nucleic acid (Aoki and Takebe, 1969). Where virus particle inocula are concerned, the polycation poly-L-ornithine (PLO) is an essential part of the inoculation mixture (Takebe and Otsuki, 1969; Barker and Harrison, 1977a). With all but a few viruses (Hibi *et al.*, 1975; Koike *et al.*, 1977), infection will only take place in the presence of a polycation such as PLO.

It is thought that the molecular weight of the polycation is important. For example, when poly-D-ornithine (mwt 13 000) was substituted for PLO (mwt 120 000) in inocula of tobacco rattle virus (TRV), little infection took place (Harrison and Mayo, 1983) compared to the normal levels achieved with PLO.

Before inoculation takes place, the virus inoculum is incubated for 5-10 minutes with the PLO and buffer (Kubo *et al.*, 1975b). Citrate and phosphate are the most common buffers used (Takebe and Otsuki, 1969; Kubo *et al.*, 1976). During this time, infectious virus aggregates are formed where the negative charge on the virus particles is neutralised to some degree (Mayo and Roberts, 1979; Motoyoshi *et al.*, 1974b). This allows the now positively charged virus particles to associate with the negatively charged cell surface.

Freshly sedimented protoplasts, observed to be more prone to infection than unwashed cells (Motoyoshi *et al.*, 1974b; Takebe, 1975), are added to the inoculum mixture and again incubated for a short length of time. This suggests that the inoculation mixture is having some effect on the surface of the protoplasts, perhaps wound formation, during this time (Kassanis *et al.*, 1977).

The pH and buffer which give optimum results vary between viruses and the source of protoplasts being inoculated. Infection is enhanced when the pH of the inoculation mixture is lowered, pH 5.0 being the optimum. A pH lower than this value promotes instability of the protoplasts (Takebe,

1975). With other viruses a higher pH is preferable, eg., TRV infection is best at a pH of about 6.0 (Kubo *et al.*, 1974).

Citrate has been the buffer most commonly used, but viruses such as tobacco mosaic virus (TMV), TRV and raspberry ringspot virus (RRSV) have been shown to be more infectious when inoculated in phosphate buffer (Motoyoshi and Oshima, 1975; Kubo *et al.*, 1976; Barker and Harrison, 1977a). Moreover, when Tris-HCl, pH 8.0, was used as buffer during protoplast inoculation, in some cases infection of a greater proportion of protoplasts was shown to take place than when phosphate buffer and a lower pH was used (Motoyoshi and Oshima, 1976; Mayo and Roberts, 1979).

When using viral nucleic acid as inoculum, PEG precipitation has proven to be a consistently successful method (Dawson *et al.*, 1978; Maule *et al.*, 1980a). This consists of virus RNA being mixed with 40% polyethylene glycol 8000 (PEG) containing 3mM $CaCl_2$ at 0°C and protoplasts then being added. A 10-fold dilution is made and after being left at room temperature for 30 min, the protoplasts are recovered by centrifugation.

The mechanism of PEG-mediated infection is unknown. It may be that its precipitating effect concentrates the RNA and protoplasts together to allow association to take place. The presence of Ca^{2+} ions are essential for infection and the dilution step which takes place after mixing of the PEG, $CaCl_2$, RNA and protoplast components is important for protoplast stability (Maule *et al.*, 1980a).

A more recently developed method of protoplast inoculation with nucleic acid is that of eletroporation (Watts *et al.*, 1987). Electrical impulses

are known to reversibly permeabilise biological membranes, allowing macromolecules to be introduced into cells. Fromm *et al.* (1985) described the transfer of DNA into plant cells by this method. As the voltage increases, so does the transfer of DNA, but the number of cells which survive the inoculation decrease.

This method is reported to have a number of advantages over the PEG procedure when inoculating with nucleic acid. These include the convenience of the technique, a lower cell toxicity (PEG is reported to be detrimental to protoplast viability) and a good efficiency of inoculation. The presence of 4mM-$CaCl_2$ in the electroporation solution is reported to increase nucleic acid transfer and protoplast survival.

1.3.3 CULTURE OF INOCULATED PROTOPLASTS

The medium used for protoplast culture is based on that described by Aoki and Takebe (1969). It contains several inorganic salts but no carbon source so that the protoplasts can neither divide nor grow cell walls. An antibiotic is generally included in the medium to prevent bacterial growth (Motoyoshi *et al.*, 1974b).

The medium varies according to the protoplast source, the one mentioned above generally being used for tobacco protoplasts.

After inoculation, the protoplasts are usually incubated at temperatures between 20-25°C under constant light conditions.

1.3.4 ADVANTAGES AND DISADVANTAGES OF PROTOPLASTS

There are several advantages and disadvantages in the use of protoplasts as an experimental system.

Advantages:

1: Protoplasts form a uniform suspension in liquid medium so can be pipetted in equal aliquots.

2: The protoplasts, when inoculated, become infected simultaneously usually with no chance of secondary infection. This suggests that subsequent virus replication is relatively synchronous.

3: Protoplasts are single cells, free from the influences of other tissues and can be cultured in well defined and easily controlled conditions.

4: Comparable and replicate samples can be taken allowing the effects of different conditions and treatments to be observed.

5: Protoplasts are easily ruptured, allowing quick extraction of viral nucleic acid and protein for further assay.

Disadvantages:

1: Different preparations of protoplasts behave differently in terms of virus multiplication.

2: Their fragility means that extreme care must be taken at all times during isolation and inoculation.

3: Protoplasts contain a fairly large quantity of indigenous metabolites which cause difficulties in interpreting results of experiments designed to monitor

the fates of particular materials synthesised at particular times (Mayo and Robinson, 1977; Harrison and Mayo, 1983).

4: The isolation procedure causes some changes in the ultrastructure of the protoplast making the cell contents slightly different from those in undisturbed leaves.

5: Isolated protoplasts contain fewer polysomes and more monosome and ribosome subunits than cells of undetached leaves. Moreover, the mRNA species extracted from protoplasts isolated from certain plants have been known to be different from those extracted from the source plant (Harrison and Mayo, 1983).

6: The plants used as material for protoplast isolation are slightly unpredictable in that protoplasts can vary quite markedly in quality and stability although isolated from leaves of similar appearance.

7: It is possible that the isolation and inoculation procedures induce the production of host proteins which act against pathogens. These may affect the multiplication of the virus.

8: The species of plant from which the protoplasts are isolated is important in subsequent virus multiplication; all viruses do not infect and multiply in all protoplasts.

1.4 RESEARCH OBJECTIVES

The main objectives of the work described in this thesis were to map the 5'-end of the subgenomic RNA of PLRV, establish a groundwork of PLRV multiplication in protoplasts and detect and identify some of the products of multiplication therein.

PLRV was known to have a subgenomic RNA of approximate size 3.4 kb (Mayo *et al.*, 1984). It had been demonstrated that the primary use for this was the expression of the coat protein gene of the virus since full-length RNA did not produce a coat-protein sized polypeptide in an *in vitro* translation system (Mayo *et al.*, 1982a). Many plant viruses are known to use this mechanism of expression, especially for coat protein genes (Morch and Haenni, 1987) but few details are understood about replication of subgenomic RNA. Several workers have described subgenomic promoters which are involved in the initiation of replication of RNA at internal sites on the genome (Marsh *et al.*, 1988; Goulden *et al.*, 1990; French and Ahlquist, 1988). These are often essential for subgenomic RNA replication. Moreover, coat protein mediated virus resistance, as described in section 1.2.4, may be increased in efficiency if the subgenomic RNA promoter sequence is included in the transforming DNA sequence (Barker *et al.*, 1992). For these reasons, the 5' end of the subgenomic RNA was mapped.

The RNA which was used in these experiments was mainly derived from PLRV-infected protoplasts. PLRV had been shown to infect protoplasts

(Barker and Harrison, 1982) but few details were known concerning the multiplication of the virus and the viral products formed in this system.

In this thesis, I will firstly discuss the RNA of the virus, in particular, the subgenomic RNA and its location on the PLRV genome. Most experiments were performed using RNA extracted from PLRV-infected tobacco protoplasts but for verification, RNA extracted from PLRV-infected plant tissue and other sources of protoplasts was also used. Comparisons are made between RNA extracted from these different sources.

PLRV multiplication in protoplasts is described and discussed at length and the effect of different environmental conditions on multiplication is reported. Protoplast inoculation and incubation is optimised to give the best yields of virus possible.

Finally, a product of PLRV multiplication in protoplasts, apparently absent in preparations of purified particles (Harrison, 1984) is described and attempts have been made to characterise it.

2. MATERIALS AND METHODS

2.1 SOURCE OF MATERIALS

COATING MATERIALS

Fluoromount - Gurr microscopy materials, BDH

Repelcote - BDH

Silane A174 - BDH

Surfasil - Pierce and Warringer

DYES AND STAINS

Bromophenol blue - BDH

Coomassie brilliant blue G - Sigma Chemical Co.

Ethidium bromide - BDH

Xylene cyanol - BDH

ELECTRICAL EQUIPMENT

ELISA plate reader - Titertek Multiskan Plus

Fluorescence microscope - Reichert

Fractionater - Isco

Hybridisation oven - Techne

Light spectrophotometer - Philips Pye Unicam SP8-500 UV/VIS

Liquid scintillation counter - 1219 Rackbeta, LKB

Mistral centrifuge - MSE

PCR machine - Intelligent Heating Block, Cambio

Semi-dry blotter - Biorad

Ultracentrifuge - Beckman L8-70M

ENZYMES

Avian Myeloblastosis Virus Reverse Transcriptase - Pharmacia

Celluclast - Novo

Cellulase "Onozuka" R-10 - Yakult Honsha Co., Ltd.

DNA Polymerase 1, Klenow fragment - Boehringer Mannheim

Macerozyme R-10 - Yakult Pharmaceutical Co., Ltd.

Moloney Murine Leukaemia Virus Reverse Transcriptase - Pharmacia

Polynucleotide Kinase, T4 bacteriophage - Pharmacia

Restriction enzymes - *Bam* H1 - Boehringer Mannheim

Eco R1 - Pharmacia

Pst 1 - Boehringer Mannheim

Sty 1 - Boehringer Mannheim

RNAse Inhibitor - Pharmacia

Taq Polymerase - Cambio

GENERAL REAGENTS

Acrylamide - DNA - Acrylogel 3 solution, acrylamide 40% (w/v), N,N-methylenebisacrylamide 3% (w/v), final ratio 29.1/0.9, BDH

Protein - Acrylogel 2.6 solution, acrylamide 40% (w/v), N,N-methylenebisacrylamide 2.6% (w/v), final ratio 37/1, BDH

Sequencing - PAGE 1 Sequencing Gel Mix Ultrapure, acrylamide 38% (w/v), N,N-methylenebisacrylamide 2% (w/v), final ratio 19/1, Boehringer Mannheim

Agarose, low melting point - BRL

Agarose NA - Sigma Chemical Co.

6-amino-n-hexanoic acid - Sigma Chemical Co.

Bovine Serum Albumen - Sigma Chemical Co.

5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt - Boehringer Mannheim

Carbenicillin Disodium Salt - Sigma Chemical Co.

d-aza-GTP - Pharmacia

d-NTPs - Pharmacia

dd-NTPs - Pharmacia

Diethanolamine - BDH

Dithiothreitol - Sigma Chemical Co.

DNA ladder - 0.92 μg/ml, fragments of sizes 1636, 1018, 517, 506, 396, 344, 298, 220, 201, 154, 134 and 75 base pairs, BRL

Ficoll 400 - Pharmacia

Fluorescein diacetate - Koch Light Labs.

Gelatin - Sigma Chemical Co.

Glycogen - Boehringer Mannheim

Herring Sperm DNA - Boehringer Mannheim

Hexadeoxyribonucleotides - Pharmacia

M13mp18 DNA - Pharmacia

Mannitol - Sigma Chemical Co.

Mercapto-acetic acid - Sigma Chemical Co.

2-Mercaptoethanol - Sigma Chemical Co.

Milk powder - Marvel, Nestle

p-Nitro blue tetrazolium chloride - Boehringer Mannheim

4-Nitrophenyl phosphate - Boehringer Mannheim

α-^{32}P-dATP - Amersham

τ -^{32}P-dATP - Amersham

Paraffin oil - BDH

Poly-L-ornithine - Sigma Chemical Co.

Potassium dextran sulphate - Meito Sangyo Co., Ltd.

Protein molecular weight markers -

bovine milk α-lactalbumin, Mr 14 200,

soybean trypsin inhibitor, Mr 20 100,

PMSF treated bovine pancreas trypsinogen, Mr 24 000,

bovine erythrocyte carbonic anhydrase, Mr 29 000,

glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle), Mr 36 000,

egg albumin, Mr 45 000,

bovine albumin (BSA), Mr 66 000,

phosphorylase b from rabbit muscle, Mr 97 400,

E. coli B-galactosidase, Mr 116 000,

myosin from rabbit muscle, Mr 205 000, Sigma Chemical Co.

Rabbit anti-mouse IgG alkaline phosphatase conjugate - Sigma Chemical Co.

RNA markers - Brome mosaic virus (BMV), tomato blackring virus (TBRV) and tobacco mosaic virus (TMV) RNAs were kindly donated by Dr. M.A. Mayo. Sizes of RNA molecules were BMV; 3236, 2864, 2109, 875, TBRV; 7356, 4662, TMV; 6397.

Sephadex G-50 - Pharmacia

Spermidine - Sigma Chemical Co.

Tween 20 - Sigma Chemical Co.

Wheatgerm carrier tRNA - Sigma

All other chemicals used were AnalaR grade from BDH.

NON-ELECTRICAL EQUIPMENT

Beta monitor - Series 900, Mini Instruments Ltd.

Centrifuge tubes - Ultraclear, 13 x 51 mm, Beckman

Chromatography paper, 3MM - Whatman

Coverslips - BDH

DE81 ion exchange paper - Whatman

ELISA plates - Multiwell immuno plate Maxisorp F96, Nunc

Flowpore disposable filters, 0.22 μm pore size - ICN Biomedicals Ltd.

Glass microscope slides - Chance Propper

Haemocytometer - Modified Fuchs-Rosenthal, 0.2 mm deep, Weber Scientific International

Nescofilm - Nippon Shoji Kaisha, Ltd.

Nitrocellulose membrane - BA-85, Schleicher and Schuell

Transilluminator camera - Polaroid CU5, 88-47

Transilluminator film - Polaroid Professional 667

X-ray film - NIF RX-100, Fuji Film Co., Ltd.

PRIMERS

Primer 1 - 3588 3' TACTCATGCCAGCACCAA 5' 3605

Primer 2 - 3521 3' GGTAAAAGTCATCGGCCA 5' 3643

Primer 3 - 3409 3' CGGTTCGTATGTGCTCAATTGTT 5' 3426

Primer 4 - 3364 5' CCGAGTGCCACCACAAAAGAACACTGA 3' 3390

The above primers were synthesized by Dr. B. Reavy using a DNA oligonucleotide synthesiser, Applied Biosystems.

M13mp18 17mer downstream primer - Pharmacia

RECOMBINANT DNA

All recombinant plasmids and M13 bacteriophages were kindly donated by Dr. M.A. Mayo

Plasmid 451 (0.24 mg/ml), pSCR 45 containing an insert of PLRV sequence from 3900 - 5987 (cut to give probe A).

Plasmid 475 (0.4 mg/ml), pSCR 45 containing an insert of PLRV sequence 3475 - 4495 (cut to give probe E).

M13 containing inserts of PLRV sequence of 3395 - 3645 and 3165 - 3375 (cut to give probes B and D respectively).

2.2 PURIFICATION OF PLANT VIRUSES

2.2.1 PURIFICATION OF PLRV VIRUS PARTICLES

Potato plants of the cultivar *Maris piper* infected with potato leafroll virus (PLRV) Scottish isolate (Tamada *et al.*, 1984) were grown in glasshouse conditions, with supplementary artificial light in winter. At approximately 4 - 8 weeks after planting, leaves and stems were removed and used for particle purification or stored at -20°C.

The purification method was as described by Harrison (1984). The leaves were ground in 0.1 M-trisodium citrate, pH 6, containing 0.5% celluclast (1500 U/g, 2ml/1g of tissue) and then stirred at 27°C for 2 hrs until the leaves were macerated. After adjusting the pH of the solution to pH 7.0 by the addition of a saturated solution of Na_2HPO_4, the suspension was emulsified with 0.67 volumes of chloroform/butanol (1/1, v/v), stirred for 20 min and centrifuged for 30 min at 3000 rpm in a MSE Mistral centrifuge. All centrifugations steps in the virus purification procedures described here were performed at 20°C.

The aqueous phase was removed and solid polyethylene-glycol (PEG) 6000 and NaCl were added to final concentrations of 8% (w/v) and 0.2 M respectively. The mixture was stirred for 1 hr at room temperature and then centrifuged for 30 min at 3000 rpm. The precipitate was resuspended in 0.02 M-phosphate buffer, pH 7.5 containing 1% Triton X-100 (v/v, 1 ml/5g tissue) and left overnight at 4°C.

The resuspended pellet was centrifuged at 10 000 rpm for 10 min and the resulting supernate was spun in a Beckman L8-70M Ultracentrifuge at 50 000 rpm for 90 min through a 20% sucrose cushion (w/v in 0.02 M-phosphate buffer, pH7.5). The pellet was resuspended in 0.02 M-phosphate buffer pH 7.5 (1 ml/25g) and left overnight at 4°C.

The virus particles were further purified by two more cycles of differential centrifugation until the pellet was suffuciently clean.

The concentration of virus in the final sample was determined from its absorbance at 260 nm. An absorbance of 8.6 (in a 1 cm path length) at this wavelength was assumed to indicate a virus concentration of 1 mg/ml (Harrison, 1984).

2.2.2 PURIFICATION OF TOBACCO RINGSPOT VIRUS PARTICLES

Tobacco ringspot virus (TRSV) particles (NJ isolate) were used to inoculate *Nicotiana clevelandii* plants. Plants were grown for approximately 25 days in the glasshouse before inoculation and were retained in these

conditions for another 12 days post-inoculation when they appeared to be showing strong symptoms of infection.

TRSV particles were purified as described by Mayo *et al.* (1982b). Infected leaves were triturated in 0.07M-sodium phosphate buffer, pH 7.0, containing 0.01M-EDTA and 0.1% mercapto-acetic acid (v/v) (2 ml/g of leaf tissue). This was filtered through muslin. Butanol was added to 8.5% (v/v) while the mixture was stirring. Stirring was continued for 20 min at room temperature and the mixture was centrifuged at 10 000 rpm for 10 min. The aqueous layer was removed and mixed with NaCl and PEG 6000 to 0.17 M and 10% (w/v) respectively. The solution was stirred at room temperature for 1 hr and centrifuged at 10 000 rpm for 10 min. The precipitate was resuspended in 0.07M-phosphate buffer, pH 7.0 (1ml/5g of leaf tissue) and left overnight at 4°C.

The resuspended pellet was centrifuged at 10 000 rpm for 10 min and the supernate was removed and centrifuged at 50 000 rpm for 2 hr. This pellet was resuspended in 0.07M-phosphate buffer at 1 ml/25g of leaf tissue. Two more rounds of differential centrifugation followed.

The concentration of virus present in the final sample was determined from its absorbance at 260 nm. The purified preparation had been determined by sucrose density gradient centrifugation to consist mainly of particles of the bottom component. At this wavelength, with a light path of 1 cm, an absorbance of 10.0 was assumed to indicate a virus concentration for the bottom component of 1 mg/ml (Stace-Smith, 1970).

2.2.3 PURIFICATION OF ARABIS MOSAIC VIRUS-LIKE PARTICLES

Plants of *N. tabacum* cv. Xanthi which had been transformed with the coat protein gene of arabis mosaic virus (ArMV) as described by Bertioli *et al.* (1991) were grown from seed in controlled conditions of 16 hrs light at 25°C and 8 hrs darkness at 20°C. These plants have been shown to contain virus-like particles which do not contain any nucleic acid (Bertioli *et al.*, 1991).

The empty virus-like particles were purified from the leaves of the transformed plants essentially as described by Harrison and Nixon (1960) with several modifications. The leaves were macerated in 0.07 M-phosphate buffer, pH 7.0, at a ratio of 2 ml buffer per 1g leaf tissue. An equal volume of butanol/chloroform (v/v, 1/1) was added and the emulsion was stirred for 10 min at room temperature. It was then centrifuged for 10 min at 10 000 rpm and the aqueous phase removed. This was centrifuged at 45000 rpm for 90 min and the pellet was resuspended in 400 µl 10 mM-phosphate buffer, pH 7.0. After overnight storage at 4°C, the suspension was centrifuged at 10 000 rpm for 10 min and the supernatant fluid loaded onto a 40% sucrose cushion made up in 10 mM-phosphate buffer, pH 7.0. This was centrifuged at 65 000 rpm for 2 hrs. The resulting pellet was resuspended in 200 µl 10 mM-phosphate buffer, pH 7.0, and stored overnight at 4°C. The suspension was centrifuged at 10 000 rpm for 10 min and the supernatant fraction was loaded onto a sucrose gradient. This had been made up approximately 18 hrs

previously and stored at 4°C. It consisted of 1.2 ml layers of 10%, 20%, 30% and 40% sucrose in 10 mM-phosphate buffer, pH7.0 (w/v) in 5 ml Beckman ultraclear centrifuge tubes (13 x 51 mm).

The gradient was centrifuged at 50 000 rpm for 45 min in a SW50 rotor in a Beckman L8-70M ultracentrifuge.

A light scattering band which was located 3.5 ml from the bottom of the gradient contained the particles and was collected by upward displacement using an ISCO fractionater.

2.3 ISOLATION AND INOCULATION OF PROTOPLASTS

Protoplasts were usually isolated from *Nicotiana tabacum* cv. Xanthi, but were occasionally isolated from *N. clevelandii* and *Chenopodium quinoa.* Tobacco plants were grown in the glasshouse at 15 - 20°C for 35 days and then transferred to a controlled environment similar to that described by Kubo *et al.*, (1975a). The conditions were illumination of 10 000 lux at plant level for 16 hrs at 25°C alternating with 8 hrs of darkness at 20°C. *N. clevelandii* plants were grown in a glasshouse at 15-20°C for 35-45 days and then transferred into the controlled environment described for growing tobacco plants where they remained for up to 10 days.

C. quinoa plants were grown in the glasshouse for about 25 days at 15-20 °C and moved into the controlled environment described above for up to 10 days.

2.3.1 ISOLATION OF TOBACCO PROTOPLASTS

Tobacco protoplasts were isolated essentially as described by Kubo *et al.* (1975b) and Barker and Harrison (1977a). All glassware used in the following procedures was siliconised by treatment with "Repelcote" and baked for 1 - 2 hrs at 80°C.

Leaves were picked from the upper region of plants which had between 6 and 8 fully expanded leaves. The lower epidermis was stripped off, the central vein was removed and the stripped leaves were cut into pieces of about 3-4 cm square. Pieces from up to 3 leaves were floated in 30 mls of maceration solution (0.25% macerozyme R-10, w/v, 0.5% potassium dextran sulphate, w/v, in 0.7 M-mannitol, pH 5.8) and vacuum-infiltrated for 30 seconds. The leaf pieces were shaken at 120 strokes/min for 3 min at 25°C, filtered through one layer of muslin and rinsed with mannitol solution. Fresh maceration solution (30 ml) was added and the leaf pieces were shaken for about a further 7 min. When light microscopy showed that some columnar cells had been released by the maceration, the leaf pieces were recovered by filtration through muslin, washed and placed in the remaining 40 ml of fresh macerozyme solution. They were then shaken at 25°C, 120 strokes/min for 1 hr. The suspension was then filtered to remove debris and the filtrate was centrifuged for 1 min at 600 rpm. The pellet of cells was gently resuspended in 50 mls of 1% cellulase "Onozuka" R-10 (w/v) in 0.7 M-mannitol, pH 5.4 and this suspension was shaken at 30 strokes/min for 60-90 min at 35°C.

The resulting protoplasts were filtered through 2 layers of muslin and washed at least 3 times by centrifugation and resuspension in 0.7 M-mannitol.

The cells were counted using a modified Fuchs-Rosenthal haemocytometer and kept at 4°C for 1 hr before inoculation (Fig 1.1).

2.3.2 ISOLATION OF PROTOPLASTS OF *NICOTIANA CLEVELANDII*

Protoplasts were isolated from *N. clevelandii* using the one step procedure of Otsuki and Takebe (1969b). In this method, the largest 2 leaves from each plant were taken after 6-10 days of growth in the controlled environment.

After the removal of the lower epidermis with forceps, leaves were cut into pieces of about 2 cm square which were floated on sterile 0.6 M-mannitol for 1 hr at 30°C with gentle agitation (approximately 40 strokes/min). The solution was replaced by 0.25% (w/v) macerozyme R-10 and 1% (w/v) cellulase "Onozuka" R-10 in sterile 0.6 M-mannitol, pH5.8, adjusted with 0.05 M-KOH. After incubation at 30°C for 90-120 min with gentle shaking (30 strokes/min) the released protoplasts were filtered through 2 layers of muslin, centrifuged for 1 min at 600 rpm and washed 3 times with 0.6 M-mannitol. The quantity of protoplasts present were determined as described above.

2.3.3 ISOLATION OF PROTOPLASTS OF *CHENOPODIUM QUINOA*

Protoplasts were isolated from *C. quinoa* essentially as described by de Varennes *et al.* (1984). The second pair of leaves from plants 15-20 cm tall with 6-8 leaves were briefly sterilised by immersion in 75% ethanol followed by washing with sterile distilled H_2O. They were lacerated and cut into approximately 1cm square pieces. These were floated on a solution of 0.6 M-mannitol for 30 min at 30°C. The mannitol solution was replaced by 50 ml 0.35% macerozyme R-10 (w/v) and 1.5% cellulase "Onozuka" R-10 (w/v) in protoplast culture medium (described in section 2.3.5) in 570 mM-mannitol. The leaf pieces were incubated for 2-3 hrs at 30°C with gentle shaking (30 strokes/min) followed by filtration through 2 layers of muslin. The filtrate was centrifuged at 600 rpm for 1 min and washed 5 times with 0.6 M-mannitol. The cells were counted using a Fuchs-Rosenthal haemocytometer.

2.3.4 INOCULATION AND CULTURE OF PROTOPLASTS

The inoculation method used was the same for the different protoplasts and viruses mentioned, with the same concentration of mannitol being used in both the isolation and inoculation procedures, i.e. 0.6M for *N. clevelandii* and *C. quinoa* and 0.7M for *N. tabacum*.

An inoculation mixture consisting of 9 ml of 0.7 M-mannitol, 1 ml of 0.05 M-potassium phosphate buffer, pH 6.0, 20 µl of poly-L-ornithine (PLO, Mwt 120 000; 1 mg/ml) and 2 µg of PLRV virus particles or 20µg of TRSV virus particles was made in a 50 ml tube. The same mixture was made in a second tube lacking only the virus component. This tube served as the mock-inoculated sample.

The mixture was left at room temperature for 10 min. After 5 min, batches of 1 x 10^6 protoplasts were centrifuged at 600 rpm for 1 min. The supernate was removed, the pellet resuspended in 10 ml of 0.7 M-mannitol and immediately tipped into the virus mixture.

This mixture was incubated at room temperature for 10 min and spun at 600 rpm for 1 min. The cells were washed once with 20 ml and twice more with 10 ml of 0.7 M-mannitol containing 0.1 mM-$CaCl_2$.

The cells were finally resuspended in 10 ml of incubation medium (0.2 mM-KH_2PO_4, 1 mM-KNO_3, 1 mM-$MgSO_4$, 10 mM-$CaCl_2$, 1 µM-KI, 0.01 µM-$CuSO_4$, 400 µg/ml carbenicillin (disodium salt), pH 5.4; sterilised through 0.22 µm pore size Flowpore filters) and incubated at 21 - 22°C with continuous illumination at 3 000 lux.

2.3.5 HARVEST OF PROTOPLASTS AND THE MEASUREMENT OF THE PROPORTION OF PROTOPLASTS INFECTED

Protoplasts were harvested by centrifugation at 600 rpm for 1 min and resuspended in a small volume of 0.7 M-mannitol. They were then counted and the rate of survival calculated.

The percentage of infected protoplasts in the sample was established by the method of Kubo *et al.* (1975), using fluorescein isothiocyanate-conjugated antibodies (supplied by Dr. P.M. Derrick).

Slides were washed in 95% ethanol, polished and coated with a very thin film of Mayer's albumen (5 ml egg white, 50 ml glycerol, 1 g sodium salicylate). A drop of protoplasts was put on the slide, spread by shaking and left at room temperature until the mannitol at the extreme edge of the drop had crystallised. The rest of the sample was then dried rapidly using a hairdryer.

Once dry, the slide was placed in 95% ethanol for 15 min, rinsed in 95% ethanol, placed in 0.01 M PBS (0.01 M-NaH_2PO_4, 0.01 M-Na_2HPO_4, 8.5% NaCl w/v, pH 7.0.) for 15 min. and rinsed in PBS. The slide was dried and 20 µl of FITC-labelled τ-globulin prepared in rabbits against PLRV, as described by Otsuki and Takebe (1969a), was placed onto the drop of cells and incubated at 37°C for 1.5 hrs in a damp chamber.

The slide was rinsed again in PBS and left in PBS for 20 min. It was then dipped in distilled H_2O and dried completely on a warm hotplate.

A drop of Fluoromount mountant was placed on the slide and a polished coverslip placed on top. The slides were examined under the fluorescence microscope (Reichert). Infected cells were bright green in colour and non-infected cells were dull green (Fig. 2.1).

An alternative method of establishing percentage infection of protoplasts in the form of immunoblotting was devised by Jung *et al.* (1991). This involved spotting about 5µl of the pelleted protoplast sample onto a small piece of nitrocellulose paper. This was left to dry for at least 15 min. The nitrocellulose paper was then placed in a solution of 5% milk powder (w/v) in TBS/Tween (0.01 M-TrisHCl, pH 7.4, 0.9% NaCl, w/v, containing 0.5 ml Tween 20 per litre) for 30 min at room temperature.

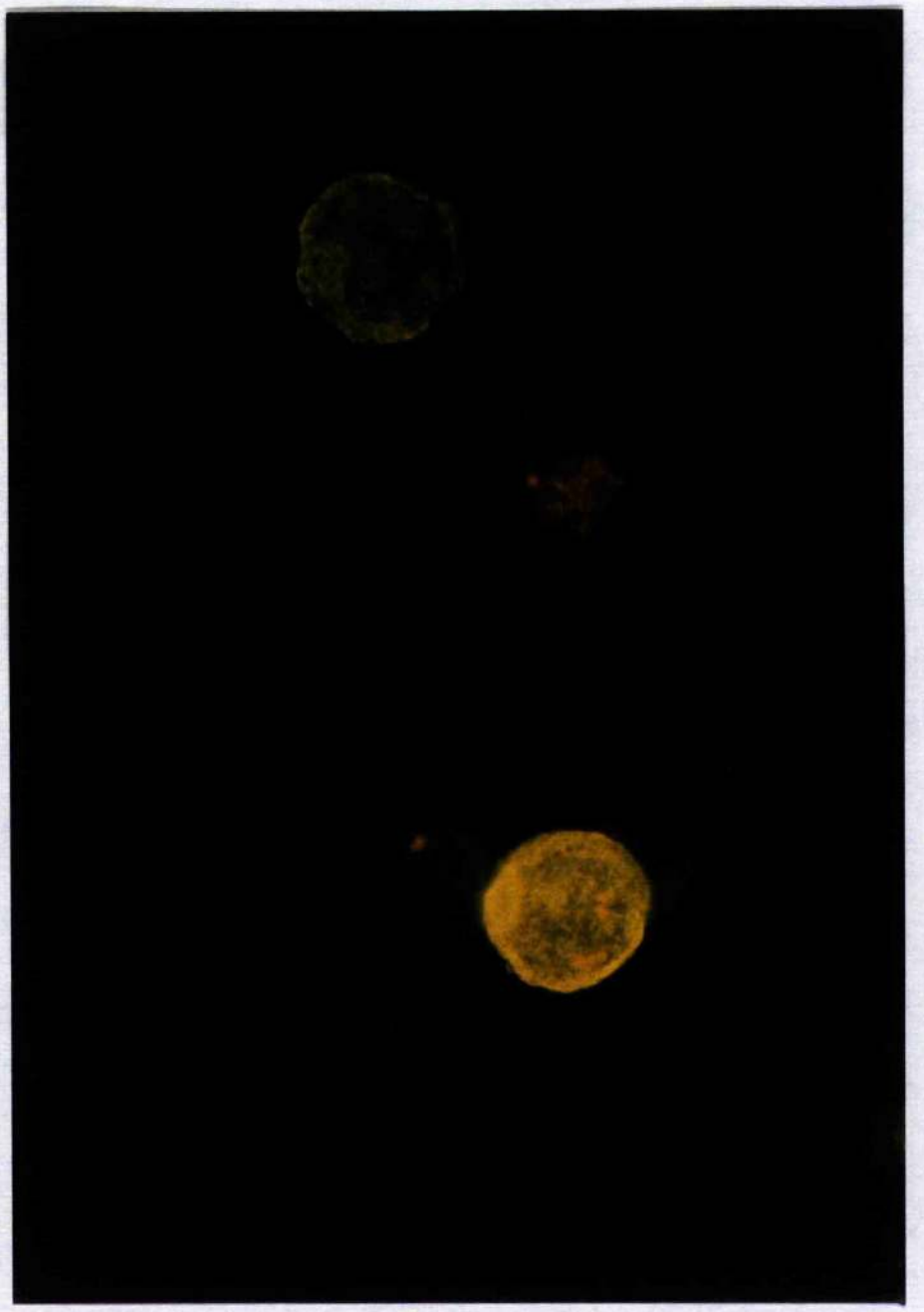
The antibody, SCR-2 (supplied by Dr. M-J. Farmer), a monoclonal antibody raised in mouse to the coat protein of PLRV, was added at a dilution of 1/1000 in fresh milk solution, to the nitrocellulose in a heat-sealable bag. This was shaken at room temperature for 2 hrs after which the blot was washed 3 times, for 5 min each, in TBS/Tween.

Rabbit anti-mouse IgG alkaline phosphatase conjugate was then added at a dilution of 1/1000 in fresh milk solution and this was shaken for 1 hr at room temperature.

The nitrocellulose was washed 3 times for 5 min each with substrate buffer (100 mM-TrisHCl, pH 9.5, 100 mM-NaCl, 5 mM-$MgCl_2$) and the colour substrates of alkaline phosphatase, BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) and NBT (p-nitro blue tetrazolium chloride), were added to the blot, each at a dilution of 1/100 in substrate buffer.

FIGURE 2.1: Protoplasts stained with FITC-labelled antibodies.

Protoplasts were isolated from plants of N. tabacum cv. Xanthi and inoculated with PLRV virus particles. A suspension of PLRV-inoculated protoplasts was placed on a glass slide which had been coated with Mayer's Albumen. The drop of protoplasts was allowed to dry and was washed several times with 95% ethanol and PBS. A volume of 20µl FITC-labelled PLRV-specific antibodies was added. Hybridisation took place at 37°C for 90 min. The glass slides were washed once more with PBS, dried and a drop of Fluoromount mountant added. A coverslip was placed on top. The fluorescent-labelled protoplasts were visualised under a fluorescence microscope (Reichert). PLRV-infected protoplasts were bright green in colour and non-infected protoplasts were dull green.



This was left at room temperature for a maximum of 10 min. During development, the colour change was checked under the light microscope and when it was judged to be strong enough, the nitrocellulose paper was rinsed under tap water for several minutes. Infected protoplasts were pink and non-infected were green.

These two techniques gave results of similar quality. Although the method of Jung *et al.* (1991) is as yet unpublished, it is the more modern and efficient technique. It does not require fluorescently-labelled antibodies nor the use of the fluorescence microscope. The materials needed for Jung's technique are commonly used in Western blotting procedures and so are available in most laboratories. In my experience, the main disadvantage in this technique is that on some occasions, a strong rapid colour reaction took place which could give a misleading positive result or false percentage of cells which had become infected.

2.3.6 ASSESSMENT OF VIABILITY OF PROTOPLASTS

Viability of protoplasts was assessed by determining their ability to take up fluorescein diacetate and hydrolyse it to release fluorescein (Widholm, 1972).

A fresh 1/50 dilution in 0.7 M-mannitol was made from a 5 mg/ml fluorescein diacetate stock solution (made up in acetone and kept at -20°C) and mixed with an equal volume of protoplast suspension in incubation

medium or in mannitol on a glass microscope slide. The protoplasts were left for 5-10 min and examined using a fluorescence microscope.

The proportion of protoplasts containing free fluorescein were determined by expressing the number of protoplasts observed under UV light as a percentage of the number observed under visible light.

2.4 GEL ELECTROPHORESIS OF NUCLEIC ACIDS AND PROTEINS

2.4.1 ELECTROPHORESIS OF RNA

RNA samples, prepared as in section 2.7.1, were loaded onto a 1.2% agarose gel containing 22% formaldehyde (v/v) and 1xMOPS buffer (0.02 M-MOPS, pH 7.0, 5 mM-sodium acetate, 1 mM-EDTA, pH 8.0). Electrophoresis in MOPS buffer was at 60-80 mA for 1.5-2 hrs.

The portion of each gel containing marker RNA was stained with a 1 μg/ml aqueous solution of ethidium bromide for 20 min and destained in H_2O for 1.5 hrs. It was photographed on the transilluminator using a Polaroid camera. The remaining portion of the gel was used for RNA transfer as described in section 2.7.2.

2.4.2 ELECTROPHORESIS OF DNA

i. Electrophoresis in low melting point agarose.

DNA which had been excised from a recombinant plasmid or prepared from a PCR reaction (sections 2.8.1 and 2.8.3 respectively) were subjected to electrophoresis in low melting point agarose to separate the DNA fragments.

The DNA was loaded onto a 1% low melting point agarose gel in 1xTBE (0.1 M-TrisHCl, 0.1 M-boric acid, 2 mM-EDTA, pH8.3) in quantities of about 30µl per well and electrophoresed for about 2 hrs at 80mA.

After rinsing the gel in water and staining with ethidium bromide, it was viewed on a transilluminator and the relevant DNA fragments excised.

ii. Electrophoresis in acrylamide

DNA was electrophoresed in acrylamide gel systems for several different purposes.

1. M13 DNA which had been labelled was separated by electrophoresis on a 6% polyacrylamide gel containing 12.6 g urea, 4.5ml acrylamide mix (38% acrylamide, w/v, 2% N,N-methylenebisacrylamide, w/v) and 1xTBE buffer in a total of 30ml. Electrophoresis was at 20mA for 1 hr after which time the appropriate band was located by brief autoradiography and excised.

2. DNA primers which had been labelled were purified by electrophoresis in an 8% sequencing gel which contained 8M-urea, 1/5 vol of acrylamide mix (38% acrylamide, w/v, 2% N,N-methylenebisacrylamide, w/v) in 1xTBE. The gel was run at 60 W for 3-4 hrs until the bromophenol

blue was about halfway down the gel. The relevant band was located by brief autoradiography and excised.

Non-radiolabelled oligonucleotide primers were purified by electrophoresis in a 20% acrylamide gel containing 8M-urea, 19% acrylamide and 1% bisacrylamide in 1xTBE. After electrophoresis at 90 mA for 3 hrs, the DNA band was located by UV shadowing. The gel was placed on a thin layer chromatography plate which fluoresces in UV light and viewed in incident UV light. DNA bands appeared as opaque shadows.

3. DNA samples which had been subjected to primer extension or dideoxy nucleotide sequencing were electrophoresed on an 8% sequencing gel at 70 W for 3 hrs. Before the gel was poured, the plates were polished thoroughly with ethanol and the large plate was coated with a solution of 5ml ethanol, 150µl 10% acetic acid and 12.5µl Silane A174. The coating was allowed to dry and the plate was again polished with ethanol. The smaller plate was coated with 2-3 ml Surfasil and polished with ethanol.

After electrophoresis, the gel was immersed in 10% acetic acid (v/v) for 20 min, washed in water for another 20 min and dried at 80°C for approximately 15 min. It was exposed to X-ray film at room temperature.

2.4.3 SEPARATION OF PROTEINS BY SDS-PAGE

SDS-PAGE was in the discontinuous buffer system of Laemmli (1970). Protein samples were electrophoresed in a 10% polyacrylamide

resolving gel with a 5% stacking gel. The resolving gel contained 10% acrylamide, 375 mM-TrisHCl, pH8.8 and 0.1% SDS. The stacking gel contained 5% acrylamide, 125 mM-TrisHCl, pH 6.8 and 0.1% SDS. Electrophoresis was at 200 V for 3 hrs in 25 mM TrisHCl, pH8.3, 250 mM-glycine, 0.1% SDS.

Protein gels were stained with a solution of Coomassie Brilliant Blue G. Approximately 0.25g of the dye was dissolved in 45% methanol (v/v) and 10% glacial acetic acid (v/v). The gel was immersed for 1 hr with shaking and the dye solution was replaced with the methanol/acetic acid mixture and allowed to destain overnight.

2.5 RECOVERY OF DNA FROM GEL MATRICES

2.5.1 RECOVERY OF DNA AFTER ELECTROPHORESIS IN LOW MELTING POINT AGAROSE GELS

After excision from the low melting point agarose gel, each gel piece containing a DNA fragment was placed in 400µl of TE (10 mM-TrisHCl, 1 mM-EDTA, pH 8.0), heated at 65°C for 5 min and extracted successively with equal volumes of phenol equilibrated with TE, phenol/chloroform (w/v, 1/1, containing 4% isoamyl alcohol, v/v, 0.1% 8-hydroxyquinoline, w/v) and chloroform. The final aqueous phase was precipitated in 2.5 volumes of

ethanol, 0.1 volume of 3 M-sodium acetate and 1µl of glycogen (10mg/ml), and stored at -20°C.

2.5.2 RECOVERY OF DNA AFTER ELECTROPHORESIS IN ACRYLAMIDE GELS

After the electrophoresis of radiolabelled M13 DNA, the relevant fragments were excised and eluted from the gel by overnight shaking in 3ml of 0.5 M-ammonium acetate, 1 mM-EDTA, 0.1% SDS (w/v) at 37°C. Efficiency of elution was verified by comparing radioactivity in the liquid with that left in the gel pieces.

The radiolabelled DNA was concentrated by ethanol precipitation in the presence of 100 µg of wheatgerm tRNA.

Purified, labelled primers were recovered by overnight shaking of the gel pieces in 800 µl of 0.3 M-ammonium acetate, 10 mM-TrisHCl, pH 7.5, at 37°C. Recovery rates for the labelled DNA were greater than 90%.

The DNA primer was then extracted successively from the eluate with equal amounts of phenol/chloroform and chloroform/isoamylalcohol. The aqueous phase was ethanol precipitated in the presence of 5 µg wheatgerm tRNA and stored at -20°C.

Purified, unlabelled primers were eluted and extracted as described above.

2.6 IMMUNOLOGICAL DETECTION OF PLRV

2.6.1 DETERMINATION OF PLRV CONCENTRATION IN INFECTED PROTOPLASTS USING DAS-ELISA

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was essentially as described by Clark and Adams (1977).

Virus antigen was trapped between τ-globulin used to coat the ELISA plate and a PLRV-specific antibody which was conjugated to alkaline phosphatase.

The ELISA plate was coated with a 1 μg/ml solution of PLRV-specific τ-globulin in carbonate buffer (15 mM-Na_2CO_3, 35 mM-$NaHCO_3$, pH 9.6). The globulin used was a gift from Dr. H. Barker and was prepared from PLRV antiserum G as described by Tamada and Harrison (1980). The plate was incubated at 37°C for 3-4 hrs, washed 3 times for 3 min in PBS/Tween (0.1 M PBS, pH 7.0 containing 0.5 ml Tween 20 per litre) and filled with 200 μl protoplast samples. These were prepared by vortexing $1x10^4$ protoplasts in 200μl extraction buffer (PBS/Tween containing 2% polyvinylpyrrolidone (PVP), w/v).

Calibration standards were made by diluting purified PLRV in PBS/Tween/PVP solution, and 200 μl samples were assayed.

After an overnight incubation at 4°C, the plate was washed as before and 200 μl of rabbit anti-PLRV antiserum conjugated to alkaline phosphatase (gift from Dr. H. Barker) and diluted 1/1000 in extraction buffer containing

0.2% ovalbumen (w/v), was added to each well. The plate was incubated at 37°C for 3-4 hrs and washed again.

300 µl of colour substrate 4-nitrophenyl phosphate at a concentration of 60 mg/100 ml of 9.7% diethanolamine (v/v), pH 9.8, was then added to each well. The absorbance, at 405 nm, of the plate was read at intervals using the Titertek Multiskan Plus ELISA plate reader.

Using the absorbances of the samples containing the known virus concentrations, a standard curve was composed (Fig. 2.2).

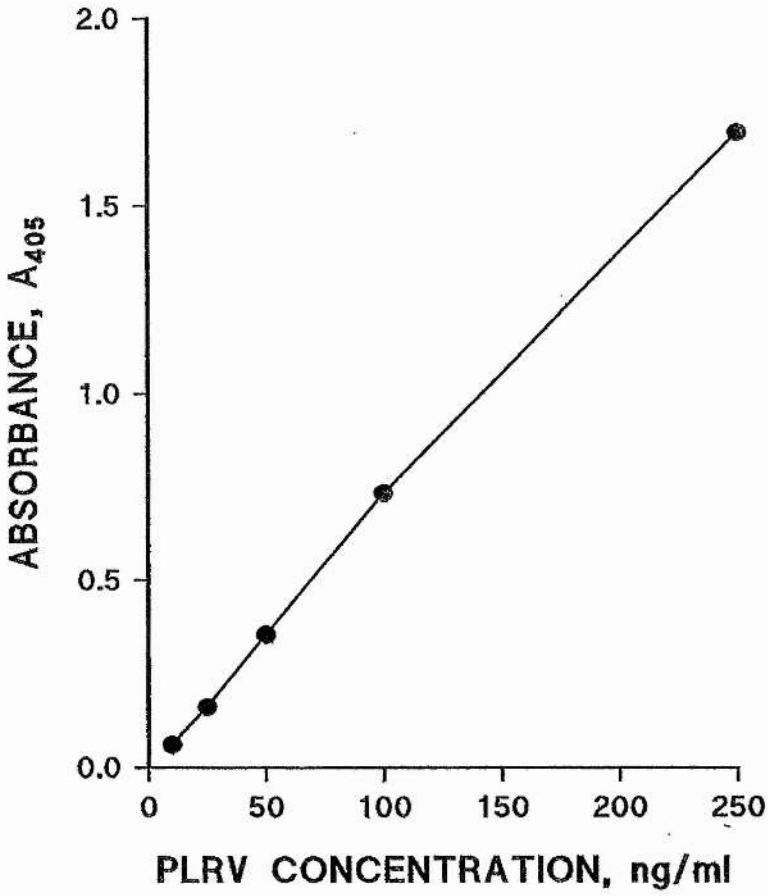
2.6.2 DETECTION OF PLRV PROTEINS IN PROTOPLASTS

i. Preparation of proteins for SDS-PAGE

Proteins were extracted from PLRV-inoculated protoplasts as described by Bahner *et al.*, (1990), by adding 50 µl of boiling protein sample buffer (125 mM-TrisHCl, pH 7.0, 150 mM-DTT, 10% SDS, w:v) to a freshly sedimented pellet of 1-2 x 10^5 protoplasts. The sample was boiled for 3 min and stored at -20°C or mixed with 0.1 of a volume of loading dye (1% bromophenol blue, w/v, 50% glycerol, v/v) and if stored frozen, boiled for a further 3 min. SDS-PAGE of these samples was carried out using the discontinuous buffer system of Laemmli (1970). Molecular weights of the viral proteins were established by comparison with protein molecular weight markers (Fig. 2.3).

FIGURE 2.2: The standard curve of PLRV virus particles as determined by ELISA.

Concentrations of 10, 25, 50, 100 and 250 ng/ml of PLRV virus particles were loaded into wells of an ELISA plate. The ELISA procedure was followed and absorbance readings taken. A plot of absorbance at 405 nm (A_{405}) versus PLRV concentration (ng/ml) was drawn. The concentration of PLRV in other samples was calculated using this curve.


2.0
1.5
1.0
0.5
0.0
ABSORBANCE, A_{405}
0
50
100
150
200
250
PLRV CONCENTRATION, ng/ml

ii. Western blotting of PLRV proteins

Proteins were transferred onto a piece of nitrocellulose paper using the semi-dry blotter.

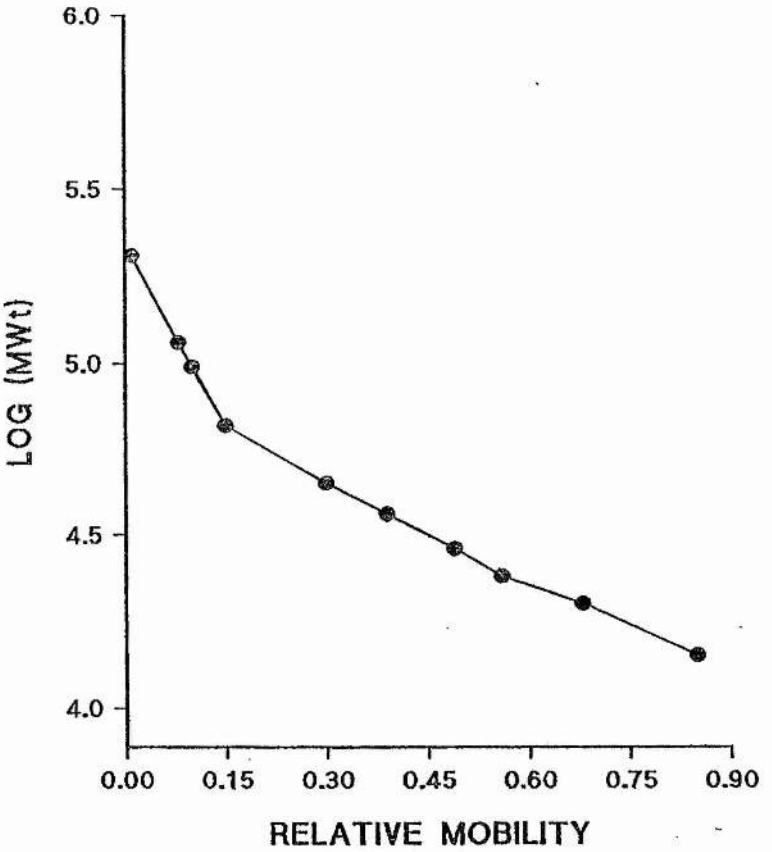
Six pieces of 3MM paper were soaked in anode solution no. 1, pH 10.4 (11 mM-TrisHCl, 20% methanol, v/v) and placed on the anode plate of the blotter. Three pieces of 3MM paper were then soaked in anode solution no. 2, pH 10.4 (0.076 mM-TrisHCl, 20% methanol, v/v) and placed on top. A piece of nitrocellulose paper was wetted in this solution and placed on top of the layers of 3MM paper. The gel was laid on top of this and pieces of Nescofilm were arranged around the edges of the layers to ensure that current passed only through the gel. Nine pieces of 3MM paper were then soaked in cathode solution, pH 7.6 (0.21 mM-6-amino-n-hexanoic acid, 20% methanol, v/v) and placed on top. Current was passed through the layers in the semi-dry blotter at 0.8 mA/cm^2 for 1 hr. The blot was removed and immersed in 5% milk solution (w/v) in TBS/Tween overnight at room temperature.

iii. Immunological detection of proteins immobilised on a Western Blot

The monoclonal antibody, SCR-2 (supplied by Dr. M-J. Farmer), raised in mice against particles of PLRV, was added to the nitrocellulose blot at a concentration of 1 µg/ml in the milk solution. This was shaken at 37°C for 2.5 hrs. The blot was then washed 5 times for 5 min each in TBS/Tween. Anti-mouse IgG alkaline-phosphatase conjugate (Sigma), raised in rabbit, was added to the blot at a 1/1000 dilution in fresh milk solution. This was shaken

FIGURE 2.3: The relative mobility of protein molecular weight markers against their log (MWt).

Protein molecular weight markers (D7, HMW; Sigma) were boiled for 3 min and electrophoresed in a 10% SDS-polyacrylamide gel. Bands were visualised by staining with a solution of Coomassie brilliant blue stain. A plot was made of relative mobility versus log (MWt). Relative mobility was calculated by dividing the mobility of the protein band with that of the dye edge (bromophenol blue).


6.0
5.5
5.0
4.5
4.0
LOG (MWt)
0.00
0.15
0.30
0.45
0.60
0.75
0.90
RELATIVE MOBILITY

at 37°C for 3 hrs and then washed 5 times for 5 min each in TBS/Tween.

The alkaline-phosphatase colour substrates, BCIP (stock solution of 15 mg/ml in N,N-dimethylformamide) and NBT (stock solution of 30 mg/ml of 70% N,N-dimethylformamide) were then added at dilutions of 1/100 each in substrate buffer and the blot was left in the dark at room temperature until sufficient colour development had taken place. It was then rinsed under tap water for several minutes, dried with a paper towel and stored in the dark at room temperature.

2.7 ANALYSIS OF VIRAL RNA

2.7.1 EXTRACTION OF TOTAL RNA FROM PLANT TISSUE AND PROTOPLASTS

RNA was extracted from plant tissue and protoplasts as according to Robinson (1982). Plant tissue extracts were ground in sterile RNA extraction buffer (10 mM-TrisHCl, pH 7.6, 50 mM-NaCl, 5 mM-EDTA, 2% SDS, w/v) in a glass homogeniser at 1 ml/0.1g or quantities of greater than $1x10^4$ protoplasts were mixed thoroughly in 200µl of the same buffer. The homogenates were heated at 60°C for 15 min. An equal volume of phenol containing m-cresol (9:1, v/v) and 0.1% 8-hydroxyquinoline (Sambrook *et al.*, 1988) was added and the mixtures were vortexed thoroughly and centrifuged at 14 000 rpm for 10 min. The aqueous phase was removed, and the original

homogenate was re-extracted with buffer. The aqueous phase from this extraction was added to the first and both were phenol-extracted again. The final aqueous phase was mixed with 2.5 volumes of 100% ethanol and 0.1 volume of 3 M-sodium acetate, pH 6.5, at -20°C overnight.

The RNA was sedimented by centrifugation at 14 000 rpm for 10 min, washed with 100% ethanol to remove all traces of SDS and phenol and dried under vacuum. The pellet was resuspended in a volume of sterile distilled water.

The RNA was again precipitated overnight in ethanol and sodium acetate at -20°C, to further purify the sample. This cycle was repeated 2 - 3 times to ensure that the RNA was free of contamination.

RNA concentration was determined by UV spectroscopy in a spectrophotometer. At 260 nm, an optical density of 1 indicates an RNA concentration of 40 µg/ml (Sambrook *et al.*, 1989). Only samples with $E_{260/280}$ > 1.8 were deemed sufficiently free of impurities to assess the concentration.

RNA was kept on ice throughout these procedures and sterile solutions were used in all experiments.

2.7.2 PREPARATION OF RNA FOR GEL ELECTROPHORESIS

The ethanol-precipitated RNA samples were washed with 100% ethanol and dried under vacuum. The RNA was resuspended in H_2O to give concentrations of 0.1µg/µl for protoplast samples and 1µg/µl for plant

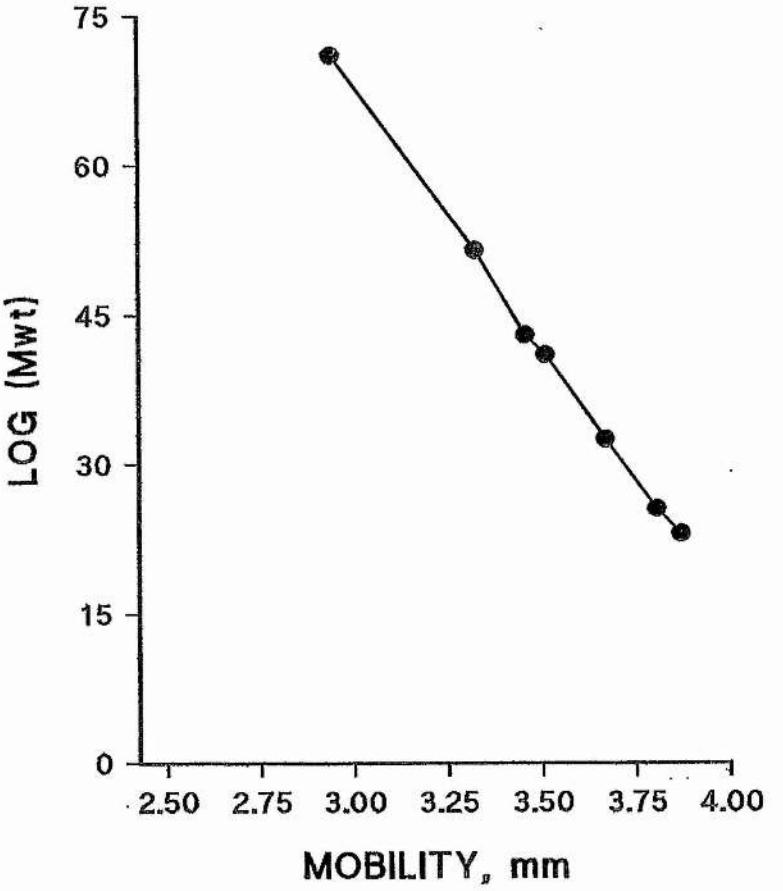
samples. Each sample, 0.5 and 5μg respectively in volumes of 5 μl, was then denatured in a mixture of 1 μl 10 x MOPs buffer, 3.5 μl 37% formaldehyde (v/v), 10 μl formamide and incubated at 65°C for 10 min. Loading buffer (1 mM-EDTA, pH 8.0, 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v), 50% glycerol, v/v), in quantities of 3μl, was mixed with each sample which was spun briefly and subjected to gel electrophoresis. Marker RNA samples contained 1 μg each of RNA extracted from particles of brome mosaic virus (BMV), tomato blackring virus (TBRV) and tobacco mosaic virus (TMV) (supplied by Dr. M.A. Mayo). Molecular weights were determined by comparison with mobility of markers plotted as log (molecular weight) against mobility (Fig 2.4).

2.7.3 NORTHERN BLOTTING OF RNA

Northern blotting was essentially according to Sambrook *et al.* (1989). After electrophoresis, the part of the gel containing the RNA samples was washed in 10 x SSC (1.5 M-NaCl, 0.15 M-trisodium citrate) for 45 min. The RNA was transferred to a piece of nitrocellulose paper by placing the gel on top of a piece of 3MM chromatography paper arranged on a tray in such a way that each end of the paper was immersed in 20 x SSC. The piece of nitrocellulose paper was cut to exactly the same size as the gel and was wetted in water for 1 min and in 20xSSC for 5 - 10 min. It was placed on top of the gel and pieces of Nescofilm were placed over the edges of the

FIGURE 2.4: The log (MWt) of TBRV, TMV and BMV RNAs against their mobility.

RNA was extracted from TBRV, TMV and BMV virus particles. Electrophoresis was in 1.2% agarose gels containing 22% formaldehyde (v/v). Bands were visualised by exposure to UV light after staining with ethidium bromide. A plot of log (MWt) of RNA versus its mobility was drawn. Using this curve, the mobility of other RNA species were calculated.


75
60
45
30
15
0
LOG (Mwt)
2.50
2.75
3.00
3.25
3.50
3.75
4.00
MOBILITY, mm

nitrocellulose/gel layers to prevent a loss of transfer efficiency caused by the movement of blotting buffer around the edges of the gel. A piece of 3MM paper, slightly larger than the gel was wetted in 20 x SSC and placed on top. A further 3 dry pieces were placed on top of that.

A layer of paper tissues about 10 cm high was added and a glass plate and weight of approximately 1 kg was placed at the very top. This was left overnight at room temperature to allow transfer of the RNA to take place.

After transfer, the piece of nitrocellulose was baked at 80°C for 2 hrs in a vacuum oven. The blot was stored in a cool, dry place until needed.

2.7.4 PREHYBRIDISATION AND HYBRIDISATION OF NORTHERN BLOTS

A prehybridisation solution containing 0.2 volumes of 10 mg/ml herring sperm DNA, 0.2 volumes of 20 x Denhardt's solution (0.4% bovine serum albumen (BSA, w/v), 0.4% Ficoll 400 (w/v), 0.4% PVP (w/v)), 0.34 volumes of H_2O and 0.06 volumes of 5% SDS (w/v), was boiled for 3 min and cooled on ice. The solution was added to the blot in a hybridisation bottle which was rotated at 65°C for 2-4 hrs.

A radiolabelled DNA probe was boiled for 3 min, snap cooled on ice and added to the prehybridisation solution already in the bottle. Hybridisation was at 65°C overnight.

The probe solution was removed from the blot and stored at -20°C. The blot was washed 4 times, each time at 65°C for 15 min in a mixture of 2 x SSC, 0.1% SDS (w/v), and then another 4 times in 0.1 x SSC, 0.1% SDS (w/v).

The blot was dried briefly, its orientation marked using ^{14}C-labelled ink and exposed to X-ray film.

2.8 SYNTHESIS OF DNA OLIGONUCLEOTIDE PROBES

Probes were made from PLRV specific DNA fragments prepared from recombinant plasmids, M13 bacteriophages or polymerase chain reaction (PCR) products (Fig 2.5).

2.8.1 PREPARATION OF DNA COMPLEMENTARY TO PLRV RNA FROM A RECOMBINANT PLASMID

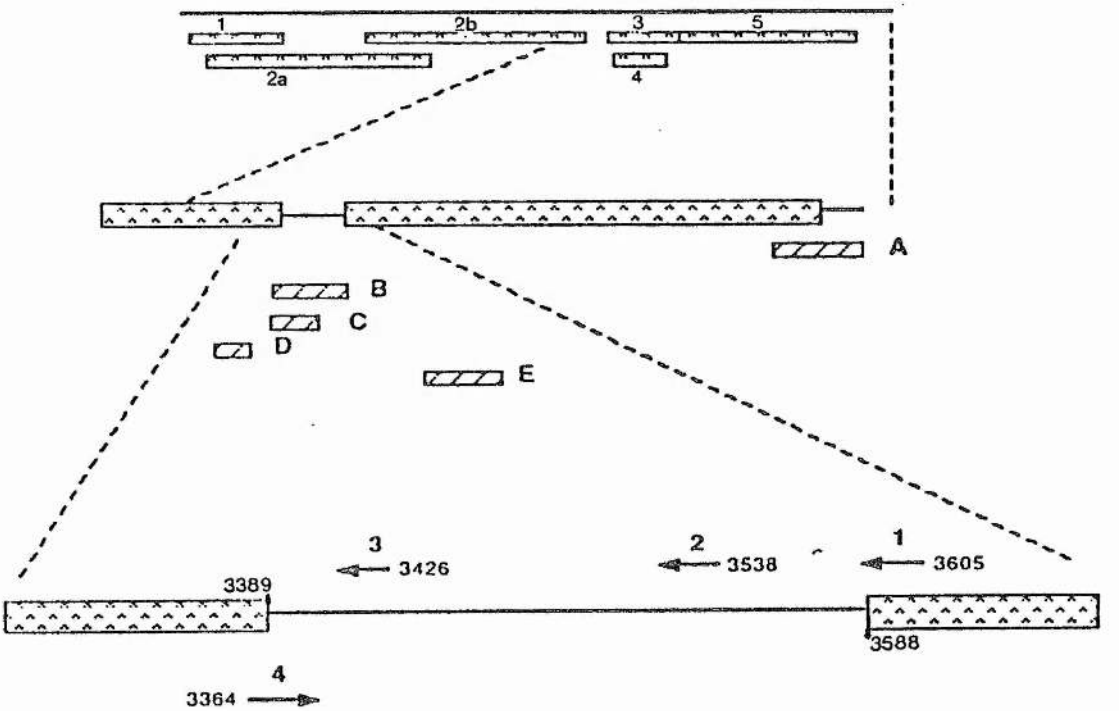
Plasmid 451 was cut with *Pst* 1 (11 U/µl) to remove DNA corresponding to PLRV sequence from position 5690 to the 3' end of the genome resulting in a piece of DNA 297 base pairs long (probe A, Fig. 2.5).

Plasmid 475 was cut at position 3941 in the PLRV sequence using *Sty* 1 (10 U/µl) and at position 4267 using *Bam* H1 (9 U/µl) to give a DNA fragment of length 326 base pairs long (probe E, Fig. 2.5).

FIGURE 2.5: The location of probes A-E and primers 1-4 on the genome of PLRV.

The upper diagram represents the entire genome of PLRV, the middle diagram represents the 3'-terminal half and the lower diagram represents nucleotides 3350-3620. The dashed lines indicate the approximate location of these regions in the PLRV RNA sequence. Dotted boxes represent ORFs, hatched boxes represent oligonucleotide probes and arrows indicate the location of primers used in primer extension and PCR experiments. Numbers indicate the nucleotide positions complementary to the 5'-ends of the primers (arrows). The probes are complementary to the following regions on the PLRV genome:

A	5690-5987
B	3395-3645
C	3364-3426
D	3165-3375
E	3941-4267


1
2b
3
5
2a
4
A
B
C
D
E
3
3426
2
3538
1
3605
3389
3588
4
3364

Ten μg of each plasmid was digested with 30 units of each enzyme at 37°C for 1.5 hrs. The DNA was electrophoresed on a 1% low melting point agarose gel (w/v). The migration of DNA fragments of known size (1kb DNA ladder; BRL) was used to locate the appropriate DNA fragment.

2.8.2 PREPARATION OF DNA COMPLEMENTARY TO PLRV RNA IN M13 BACTERIOPHAGE

DNA fragments were excised from DNA derived from recombinant bacteriophage M13 as described by Sambrook *et al.* (1989) to give probes B and D (Fig. 2.5).

A volume of 1.6 mls of 2 x TY medium (1.6% bacto tryptone, w/v, 1% yeast extract, w/v, 0.5% NaCl, w/v) was inoculated with 16 μl of an overnight culture of *Escherichia coli* DH5αF'. The M13 culture was added to a dilution of 1/100 and shaken at 37°C for 5 hrs. Cells were then removed by centrifugation for 5 min at 14 000 rpm at room temperature and the supernatant fluid was transferred into a fresh tube and centrifuged for a further 5 min at 14 000 rpm to ensure that all the *E. coli* cells had been removed.

The supernatant fluid was removed and added to 200 μl of 20% PEG 6000 (w/v), 2.5 M-NaCl. This was shaken and left to stand for 15 min. It was then centrifuged as before and the supernatant fraction discarded. The tube was spun again for 2 min and all the remaining supernatant fluid was

removed with a drawn out Pasteur pipette. Any traces of PEG on the mouth of the tube were removed with a tissue.

The pellet was resuspended in 100 µl of TE and the solution was extracted with 50 µl of TE-saturated phenol. The mixture was vortexed for 15 - 20 secs and left for 15 min. It was vortexed for a further 15 secs and centrifuged for 3 min. The aqueous layer was extracted successively with 150 µl each of phenol/chloroform and chloroform and then precipitated in 2.5 volumes of ethanol, 0.1 vol. of 3 M-sodium acetate, pH 6.0 and 1 µl glycogen (10 mg/ml).

The DNA was centrifuged for 10 min at 14 000 rpm and washed with 500 µl of ethanol. The dried pellet was redissolved in 50 µl of TE and stored at -20°C. Probe DNA was excised after labelling as described in 2.9.2.

2.8.3 PREPARATION OF DNA COMPLEMENTARY TO PLRV RNA BY PCR

PLRV-specific oligonucleotide primers were used to make polymerase chain reaction (PCR) products corresponding to parts of the PLRV genome.

First strand cDNA was made and then amplified essentially as described by Natsuaki *et al.*, (1991).

RNA which had been extracted from PLRV-infected tobacco protoplasts was washed, dried and resuspended at a concentration of 1µg/5µl H_2O. This was added to 1 µl of 10 x PCR buffer (50 mM-KCl, 10

mM-TrisHCl, pH 8.4, 1.5 mM-$MgCl_2$, 20 µg/ml gelatin), 2 µl of a 1/5 dilution of 2 mM-dNTPs and 1 µl of primer 1 (1µg/µl, Fig 2.4). The mixture was heated at 65°C for 2 min and slowly cooled to 42°C. RNAse Inhibitor (0.5 µl at 29 U/µl) and 1 µl of Moloney Murine Leukaemia Virus (MoMLV) reverse transcriptase (19 U/µl) were added and the mixture was incubated at 42°C for 2 hrs.

The mixture was then added to 10 µl of 10 x PCR buffer, 10 µl of 1/5 dilution of 2mM-dNTPs, 1 µl each of primers 2 and 4 (1µg/µl, Fig. 2.4), 68 µl of ddH_2O and 0.5 µl *Taq* polymerase (5U/µl). A drop of paraffin oil (50µl) was placed on top of the liquid to prevent evaporation.

The tube containing the PCR mixture was placed in the PCR machine on the following programme; 95°C for 1.5 min., 55°C for 1.5 min., 72°C for 2.5 min. and 72°C for an extra 5 min. This cycle was repeated 30 times.

The liquid paraffin was removed and the products electrophoresed on a 1% low melting point agarose gel. Probe C was synthesised in this manner (Fig. 2.5).

2.9 LABELLING OF DNA PROBES

2.9.1 LABELLING OF DOUBLE-STRANDED DNA PROBES

Double-stranded DNA fragments were labelled according to the method of Feinberg and Vogelstein (1983, 1984).

Approximately 0.1-0.25 μg of DNA recovered from a restriction enzyme digestion or PCR product synthesis was sufficient to be radiolabelled and used in subsequent procedures.

The DNA was resuspended in 20 μl of H_2O and boiled for 3 min then cooled on ice. The following mixture was then made; 16.5 μl H_2O, 10 μl oligo-labelling buffer

(oligonucleotide labelling buffer is a mixture of the following components in the ratio of 100:250:150, A:B:C.

solution O: 1.25 M-TrisHCl, pH 8, 0.125 M-$MgCl_2$.

solution A: 1ml solution O, 18 μl 2-mercaptoethanol, 5 μl of each of 0.1 M dCTP, TTP, dGTP.

solution B: 2 M-Hepes, pH 6.6.

solution C: hexadeoxyribonucleotides evenly suspended in TE at 90 OD units/ml at A_{260} nm),

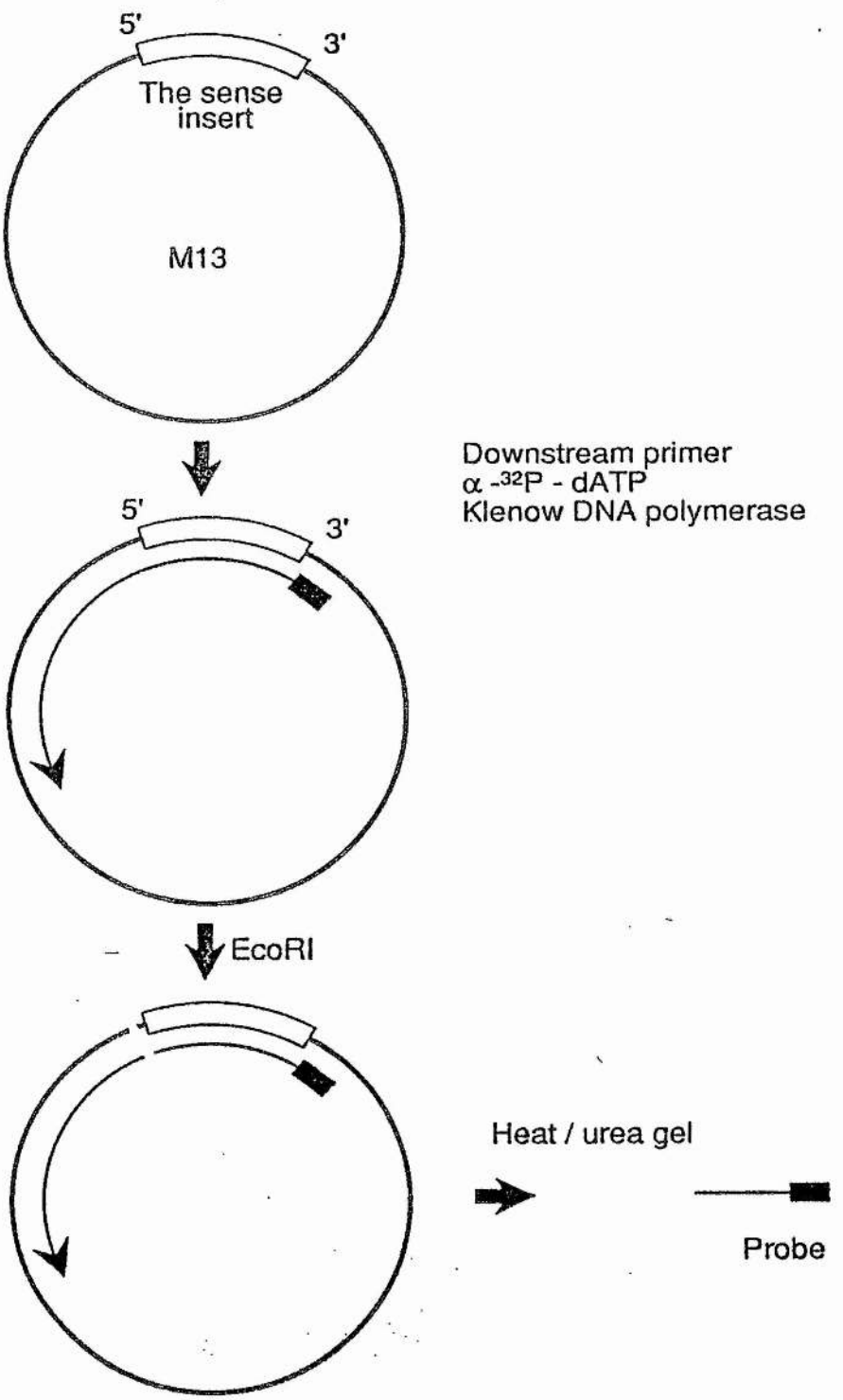
2 μl 10mg/ml BSA, 20 μl denatured DNA, 1 μl ^{32}P-dATP (10 μCi/μl, specific activity 3000 Ci/mmol), 0.5 μl DNA polymerase 1, Klenow fragment (5U/μl). This was left to incubate overnight at room temperature.

The incorporation of the ^{32}P-dATP into the DNA was determined as the proportion of the dATP used that could not be washed off DEAE-cellulose filter paper with 0.5 M-Na_2HPO_4. A sample of 5μl of a 1/20 dilution was spotted onto each of two DE81 filter discs. One disc was washed 10 times in 0.5 M-Na_2HPO_4, once briefly in water and twice in 95% ethanol (v/v). Both discs were dried and placed in scintillation counting bottles with 1 ml of toluene containing 0.5% 2,5-diphenyloxazole (PPO, v/v)

FIGURE 2.6: The synthesis and labelling of a single-stranded DNA probe.

from Barker *et al.*, 1985.

A downstream primer was used to prime synthesis of DNA on an M13 clone containing a plus insert of PLRV. Synthesis was in the presence of Klenow DNA I Polymerase, dCTP, dGTP, TTP and ^{32}P-dATP. After synthesis, the Klenow enzyme was heat-killed and DNA was digested with *Eco* R1. The desired DNA fragment was separated in a polyacrylamide gel, located by brief autoradiography, excised and eluted.


5'
3'
The sense insert
M13
Downstream primer
α -32P - dATP
Klenow DNA polymerase
5'
3'
EcoRI
Heat / urea gel
Probe

and counted in a LKB Rackbeta liquid scintillation counter.

2.9.2 LABELLING OF SINGLE-STRANDED DNA PROBES

Single-stranded DNA probes were labelled according to Barker *et al.* (1985, Fig 2.6).

Approximately 5µg of the recovered M13 DNA was boiled for 2 min with 25 ng of an M13mp18 17mer primer in 17 µl of 10mM-TrisHCl, pH 7.5, 10 mM-$MgCl_2$, 50mM-NaCl and then cooled to room temperature over 30 min. After the addition of 1 µl of 100 mM-DTT, 2 µl of a mixture of 2.5 mM-dGTP, TTP, dCTP, 2 µl of ^{32}P-dATP (10 µCi/µl, specific activity 3000 Ci/mmol) and 2 µl of DNA polymerase 1, Klenow fragment (5U/µl), the solution was incubated at 37°C for 20 min. The radioactive nucleotide was chased with 2 µl of 2.5 mM-dNTPs and incubated at 37°C for a further 20 min. The mixture was heated at 65°C for 5 min and cooled on ice.

The insert complementary to the PLRV sequence was excised with *Eco* R1 (2.5 µl of 25 U/µl) in 0.5 µl of 100 mM-DTT, 3 µl H_2O and 3.5 µl of 10 mM-TrisHCl, pH 7.5, 100 mM-NaCl, 10 mM-$MgCl_2$, 1 mM-2-mercaptoethanol, 100 µg/ml BSA. This was incubated at 37°C for 2 hrs.

Digestion was stopped and the nucleic acids were denatured by the addition of 35 µl of 95% formamide (v/v), 10 mM-EDTA, pH 8.0, 0.02%

bromophenol blue (w/v), 0.02% xylene cyanol (w/v) and boiling for 4 min followed by snap-cooling on ice.

The nucleic acids were then separated by electrophoresis on a 6% polyacrylamide gel.

2.10 LABELLING OF DNA PRIMERS

Primers were 5'-end labelled as according to Sambrook *et al.* (1989), with some slight modifications.

500 ng of the oligonucleotide primers 1, 2 and 3 (Fig. 2.5) were mixed separately with 1 µl of 10 x T4 bacteriophage polynucleotide kinase buffer (0.5 M-TrisHCl, pH 7.6, 0.1 M-$MgCl_2$, 50 mM-DTT, 1 mM-spermidine, 1 mM-EDTA, pH 8.0), 4µl H_2O, 4 µl τ-^{32}P-ATP (370 MBq/ml, specific activity >5000 Ci/mmol) and 0.5 µl T4 bacteriophage polynucleotide kinase (10 U/µl). This was incubated at 37°C for 30 min and stored at -20°C.

2.11 PRIMER PURIFICATION

Synthetic oligonucleotide primers were purified by two methods.

2.11.1 PURIFICATION OF RADIOLABELLED PRIMERS BY GEL ELECTROPHORESIS

After radiolabelling, an equal quantity of loading buffer (80% formamide, v/v, 1.6 mM-EDTA, 4 mM-NaOH, 1 mg/ml each of bromophenol blue and xylene cyanol) was added to the primer, boiled for 2 min and cooled on ice. It was then loaded onto several tracks of an 8% sequencing gel and subjected to gel electrophoresis.

2.11.2 PURIFICATION OF NON-RADIOLABELLED PRIMERS BY GEL ELECTROPHORESIS

Non-radiolabelled oligonucleotide primers were purified by electrophoresis on a 20% acrylamide gel with 5% bisacrylamide cross link followed by UV shadowing to locate the DNA band.

A pellet of about 40 µg DNA was resuspended in 10 µl of loading dye (95% formamide, v/v, 20 mM-EDTA, 1 mg/ml each of bromophenol blue and xylene cyanol), heated to 90°C for 10 min, cooled on ice and loaded into three lanes each 3 mm wide.

2.12 PURIFICATION OF DNA OLIGONUCLEOTIDE PROBES

Radioactively labelled DNA oligonucleotide probes were purified by the spin column method.

Columns comprised a 1 ml syringe containing Sephadex G-50 in 1xTE retained by a glass wool plug. Columns were spun in glass tubes at 6000 rpm for 4 min. to pack the column and were topped up with Sephadex. 100 µl of TE was added to the top of the syringe and it was placed in a lidless eppendorf tube in the test tube and spun again. This was continued until 100 µl was retrieved in the tube when 100 µl of TE was added. 100 µl of labelled probe was added and spun through the column. Any unincorporated nucleotides which were present were retained in the column and labelled DNA was collected in the tube. The DNA was ethanol precipitated and stored at -20°C.

2.13 PRIMER EXTENSION

The primer extension procedure was basically as described in Sambrook *et al.* (1989) with several modifications.

Samples of 5 µg of protoplast RNA, mock and PLRV-infected, or 1 µg of PLRV virus particle RNA was resuspended in 7 µl water and boiled with 1µl of a 5'-end labelled oligonucleotide primer (50ng/1µl) for 2 min.

After the addition of 2 µl of 80 mM-TrisHCl, pH 8.3, 0.27 M KCl, 20 mM-DTT, 40 mM-$MgCl_2$ the mixture was heated at 50°C for 20 min and left to cool at room temperature for 15 min. Reverse transcriptase buffer (86 µl of 20 mM-TrisHCl, pH 8.3, 67.5 mM-KCl, 5 mM-DTT, 10 mM-$MgCl_2$, 1 mM-dNTPs) and 4 µl (2 U) of avian myeloblastosis virus reverse transcriptase were added and the mixture was incubated at 42°C for 1.5 hrs.

Water was added to bring the volume to 200 µl and the mixture was extracted successively with equal volumes of phenol/chloroform (1/1, v/v) and chloroform/isoamyl alcohol and the extract was mixed with 2.5 volumes of ethanol.

After 30 min at -70°C, precipitated material was recovered, dried and dissolved in 50 µl of 0.3 M-NaOH. This was incubated at 65°C for 30 min and was neutralised with 60 µl of 1 M-TrisHCl, pH 7.5. After the addition of 2.5 µg of carrier wheatgerm tRNA, DNA was recovered by ethanol precipitation overnight at -20°C.

The DNA was washed with 100% ethanol, dried and dissolved in 4 µl of loading buffer (20 mM-NaOH, 8 mM-EDTA, 80% formamide, v/v, 1 mg/ml each of bromophenol blue and xylene cyanol). This was boiled for 2 min and loaded onto a 8% sequencing gel. After electrophoresis and subsequent autoradiography, bands were present where the cDNA extension of the labelled primer had stopped due to the reverse transcriptase enzyme reaching the end of the RNA template.

The sizes of the oligonucleotides formed by transcription to the end of the RNA template was determined by comparison with the products of

dideoxy sequencing of a DNA insert in the M13 bacteriophage co-electrophoresed with the primer extension products.

2.14 DIDEOXYNUCLEOTIDE SEQUENCING OF M13 DNA

The sequence procedure used was the dideoxy chain termination method as described by Sanger *et al.* (1977).

Approximately 0.25 μg of the M13 DNA preparation in 5 μl TE was added to 1 μl (2.5 μg/ml) of primer, 1 μl of 100 mM-TrisHCl, pH 8.5, 100 mM-$MgCl_2$ and 3 μl H_2O. This mixture was heated at 65°C for at least 10 min and slowly cooled to 42°C over 30 min.

The following ddNTP mixes were made up :

ddATP (0.1 mM-dTTP, 0.1 mM-dCTP, 0.1 mM-d-azaGTP, 0.08 mM-ddATP, 5 mM-TrisHCl, pH 8.0, 0.1mM-EDTA),

ddCTP (0.1 mM-dTTP, 0.01 mM-dCTP, 0.1 mM-d-azaGTP, 0.1 mM-ddCTP, 5 mM-TrisHCl, pH 8.0, 0.1mM-EDTA),

ddGTP (0.1 mM-dTTP, 0.1 mM-dCTP, 0.0075 mM-d-azaGTP, 0.12 mM-ddGTP, 5 mM-TrisHCl, pH 8.0, 0.1mM-EDTA),

ddTTP (0.005 mM-dTTP, 0.1 mM-dCTP, 0.1 mM-d-azaGTP, 0.5 mM-ddTTP, 5 mM-TrisHCl, pH 8.0, 0.1mM-EDTA).

A 2 μl aliquot of each mix was added to individual tubes and 2 μl of isotope mix (3 μl 2.5mM-dATP, 1 μl ^{32}P-dATP, 1 μl DNA Polymerase 1, Klenow fragment (5U/μl), was then added to the M13 DNA tube and 2.5 μl

from this was added to each ddNTP tube. These were left at room temperature for 15 min and 2 µl of chase solution (2.5 mM-dATP, 2.5 mM-dCTP, 2.5 mM-dGTP, 2.5 mM-TTP) was added. This was also left at room temperature for 15 min and 4 µl of stop solution (95% formamide, v/v, 20 mM-EDTA, 1 mg/ml each of bromophenol blue and xylene cyanol) was added and the tubes were boiled for 2 min. The sequenced DNA was loaded onto a 8% sequence gel and electrophoresed as described for the primer extension products.

2.15 SUCROSE GRADIENT CENTRIFUGATION OF PROTOPLAST LYSATES

Protoplasts were recovered from incubation medium by centrifugation at 600 rpm for 1 min. The pellet was resuspended in 400 µl of 10mM-phosphate buffer, pH 7.0 and the suspension was mixed by vortexing. After centrifugation at 10 000 rpm, for 10 min, the supernate was removed and 200 µl was layered on to a 10 - 40% sucrose gradient.

Gradients were centrifuged as described previously.

2.15.1 FRACTIONATION AND ASSAY OF SUCROSE GRADIENTS

After centrifugation, the gradients were fractionated by upward displacement using an ISCO-fractionater and collected in 7 drop fractions. A portion of each fraction was assayed for PLRV content by DAS-ELISA following dilution to 200 µl in ELISA extraction buffer.

The pellet which remained after centrifuging the lysed protoplasts, resuspended in 200 µl of extraction buffer and 100µl of the unfractionated supernate diluted with 100µl of extraction buffer were also assayed by ELISA.

A 3 µl volume of each fraction was spotted onto a piece of nitrocellulose paper which had been wetted in 20xSSC. This was allowed to air dry for 30 - 60 min, was baked at 80°C for 2 hrs under vacuum and stored in a cool, dry place until hybridisation could proceed.

Lastly, the remains of the fractions were used for RNA extractions. The components of RNA extraction buffer were added to a tube containing several of the fractions bulked together and the normal procedure followed.

3. STUDIES OF THE PLRV SUBGENOMIC RNA

3.1 INTRODUCTION

A subgenomic RNA is defined as being related in sequence to the viral genomic RNA and having the ability of mRNA to express one or more viral proteins during *in vitro* translation (Palukaitis, 1984). Partial transcription to yield a subgenomic RNA is a strategy commonly used by plant viruses, many of which are positive-strand RNA viruses, to express part of the viral genome (Davies and Hull, 1982). Usually the open reading frames (ORFs) nearest the 3'terminus of the genome are expressed by translation of a subgenomic RNA rather than of intact genomic RNA. With some viruses such as BMV and turnip yellow mosaic virus (TYMV; Peden and Symons, 1973; Bruening *et al.*, 1976; Gargouri *et al.*, 1989), the subgenomic RNA is encapsidated; for others such as TMV, the subgenomic RNA is found only in virus-infected plant cells (Davies and Hull, 1982).

To date, all the definitive luteoviruses for which a genome organisation has been established have been shown to produce at least one subgenomic RNA in infected cells (Table 1.3b). Mayo *et al.* (1984) found one subgenomic species of RNA, with an estimated size of 3.4 kb, in PLRV-infected protoplasts. Two less abundant RNA species of size 4.9 and 2.1 kb were also observed but were thought to be ribosomal species. Furthermore, Tacke *et al.* (1990) found one PLRV subgenomic RNA of size 2.3 kb.

In this chapter, using probes which were complementary to different parts of the PLRV genome as detailed in chapter 2 (Fig. 2.5), experiments are described in which RNA was detected in infected protoplasts and plant tissue (Figs. 3.1, 3.4, 3.5 and 3.6). The detection and mapping of the subgenomic RNA was performed and a potential subgenomic RNA promoter found.

The electrophoretic migration of PLRV RNA extracted from different species of plant and protoplast source plant was compared to determine if the results observed in protoplast extracts were also observed in plant extracts. Moreover, comparisons were made to detect differences which may have arisen in infected plants, both hosts and test plants, known to have different levels of resistance to PLRV infection. Extracts from PLRV-infected transgenic potato and tobacco plants were also analysed to establish if any differences in virus multiplication existed.

Further analysis was performed on RNA extracted from different tissues within a young and an old potato plant to compare RNA content and to attempt to determine where the most virus multiplication was taking place.

When discussing the northern blots described in this chapter, it has been assumed that hybridisation was constant across the area of the blot. This is because the bands seen on the blot were not RNA, but the labelled DNA probe which hybridised, in most cases specifically, to the PLRV RNA and was subsequently detected by autoradiography. Therefore the quantitative differences in RNA can be assumed to be accurate on a single blot. However, differences between RNA on separate blots cannot be assumed to be accurate

and have not been compared. The results described here were obtained several times with separate blots.

3.2 DETECTION OF SUBGENOMIC RNA

Northern blot hybridisation with probe E detected two species of RNA in extracts from PLRV-infected potato plants (Fig. 3.5, lane 1) and PLRV-inoculated protoplasts (Fig 3.1, lane i). The slower migrating band corresponded to a molecule of size 6.0 kb, the genomic RNA, and the faster migrating band to a smaller molecule of approximate size 2.7 kb, presumably the non-encapsidated subgenomic RNA observed by Mayo *et al.* (1984) and Tacke *et al.* (1990). Both RNA species were also detected by several other probes (probes A, B and C, Fig. 3.1).

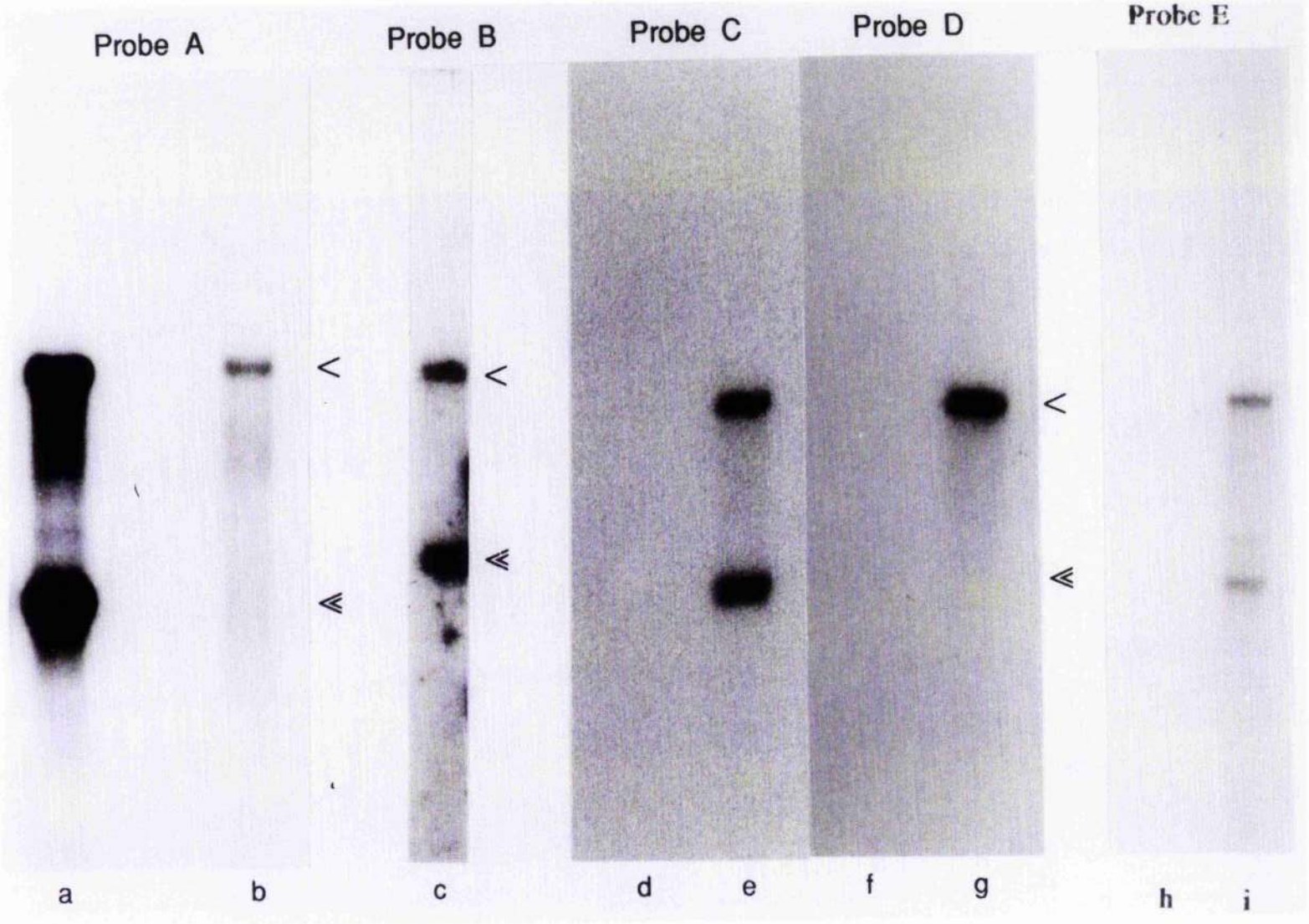
In RNA extracted from PLRV virions, only one band could be detected, the 6.0 kb genomic band (Fig. 3.1, lane b). This supports the evidence of Mayo *et al.* (1984) that the subgenomic RNA is not encapsidated.

Both RNAs were detected in PLRV-infected tissues of other plant species and protoplasts (Table 3.1) using northern blot hybridisation.

In some RNA samples, bands other than the genomic and subgenomic RNA bands were detected but when gels containing these samples were stained prior to blotting, these bands were visible. From their size, these bands corresponded to plant ribosomal RNA which presumably had bound

FIGURE 3.1: Detection of PLRV RNA species in infected protoplasts with probes complementary to different areas of the PLRV genome.

Blots were made in 5 separate experiments and hybridised with probes A to E respectively. Samples were RNA from PLRV particles (lane b), PLRV-infected protoplasts (lanes a, c, e, g and i) and buffer-inoculated protoplasts (lanes d, f and h). Single arrow heads indicate genomic RNA, double arrow heads indicate subgenomic RNA.


Probe A
Probe B
Probe C
Probe D
Probe E
a
b
c
d
e
f
g
h
i

non-specifically to the radiolabelled DNA probe (see section 3.9, non-specific hybridisation).

3.3 SIZING OF SUBGENOMIC RNA

Hybridisation with probe A (Fig 3.1, lane a) detected both RNA species, suggesting that the subgenomic RNA terminates within about 30 nucleotides of the 3'end of the genome RNA, and is presumably 3' co-terminal with the genomic RNA. A 2.7 kb molecule terminating at this position would have a 5' end at about nucleotide 3200. However, when probe D was used in Northern blot analysis, the subgenomic RNA was not detected (Fig 3.1, lane g). Probe D terminates at nucleotide 3375 so the subgenomic RNA must begin close to or 3' of this point.

When probe C was used, both genomic and subgenomic RNAs were detected (Fig 3.1, lane e). This suggested a more precise location for the 5' end of the subgenomic RNA. Probe C extends from position 3364 to 3538, so its 3' end is 51 bases upstream of the start of the coat protein gene (and 11 bases upstream of the position proposed by Tacke *et al.* (1990) for the 5' end of the subgenomic RNA). These results suggested that the 5' end of the subgenomic RNA is between nucleotides 3370 and 3500 (Miller and Mayo, 1991).

3.4 PRECISE MAPPING OF THE 5' TERMINUS OF THE SUBGENOMIC RNA BY PRIMER EXTENSION

The first primer extension experiments were done using primer 1 (Fig 2.5), which is complementary to 18 nucleotides at the 5' terminus of the coat protein gene. Products primed on RNA from infected protoplasts produced several bands, but none were produced when RNA from healthy protoplasts was used (Fig. 3.2(a)).

Although virus particles do not contain subgenomic RNA, extension from primers annealed to RNA extracted from PLRV particles yielded several bands. These bands were also detected in analyses of samples primed on RNA from PLRV-infected protoplasts and are presumably products formed by DNA elongation stopping at positions of strong secondary structure. One prominent band of approximately 205 nucleotides was consistently present in extension products primed on RNA from infected protoplasts, but was not found in those primed on virus particle RNA.

This suggested that this band represented extension to the end of the subgenomic RNA and therefore that the end of this molecule was located more than 200 nucleotides upstream of the initiation codon of the coat protein gene. Primer extensions of such a length are known to result in stops (Sambrook *et al.*, 1989) and therefore a second primer (primer 2), complementary to the sequence 47 nucleotides upstream of the coat protein AUG was used. If the estimate for the size of the subgenomic RNA established by Tacke *et al.* (1990) was accurate, primer 2 would only

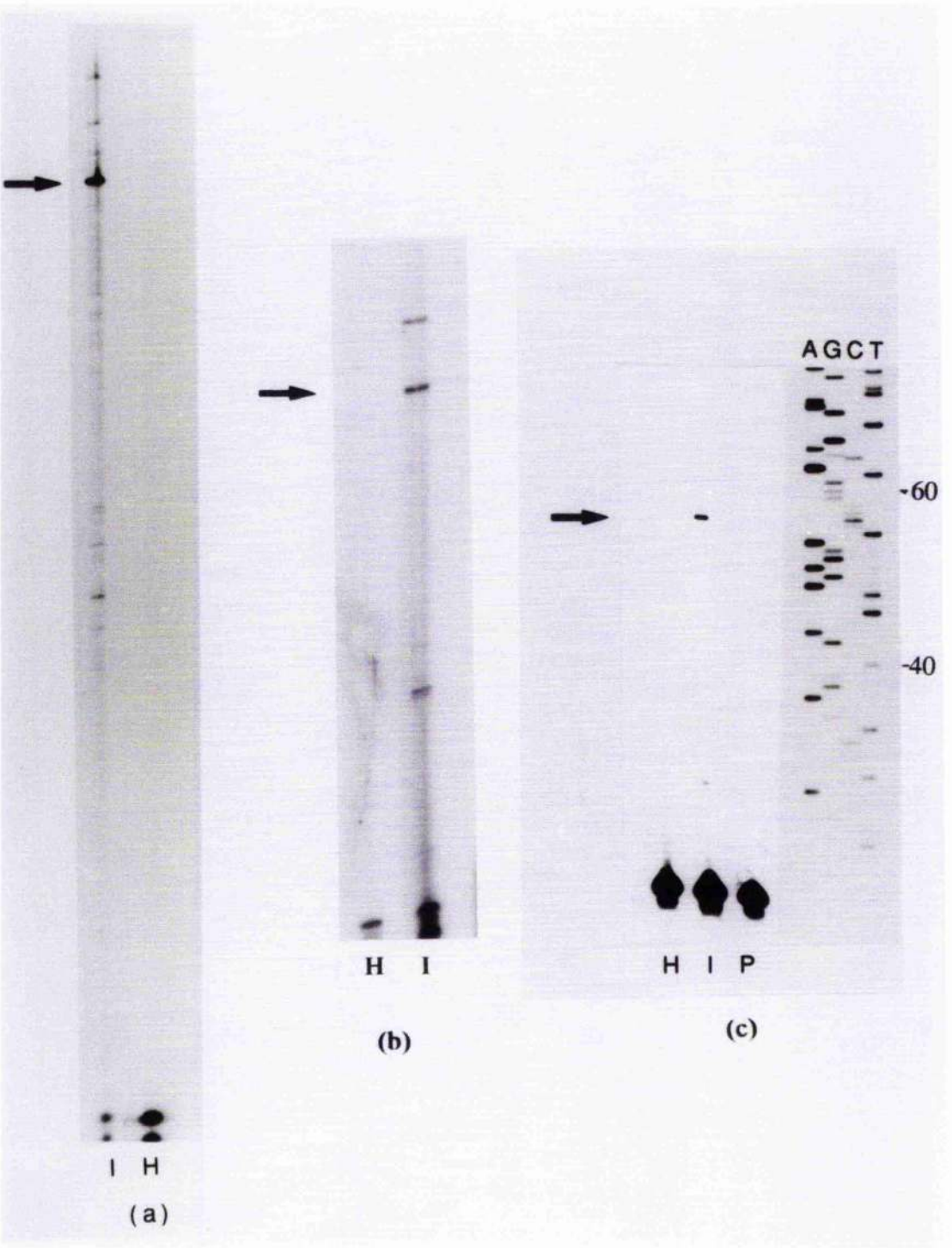
FIGURE 3.2: Precise mapping of the 5'-terminus of the subgenomic RNA of PLRV by primer extension.

a. Extension from primer 1. Samples were RNA from PLRV-infected protoplasts (lane I) or buffer-inoculated protoplasts (lane H). The arrow indicates the most prominent infection-specific product.

b. Extension from primer 2. Samples were RNA from PLRV-infected protoplasts (lane I) or buffer-inoculated protoplasts (lane H). The arrow indicates the most prominent infection-specific product.

c. Extension from primer 3. Samples were RNA from buffer-inoculated protoplasts (lane H), PLRV-infected protoplasts (lane I) or RNA from purified virus particles (lane P). The arrow indicates the most prominent infection-specific product.

Lanes A, G, C and T indicate products of dideoxynucleotide sequencing of M13mp18 DNA. Numbers on the right are the sizes in nucleotides of the oligonucleotide bands indicated.


I H
(a)
H I
(b)
A G C T
-60
-40
H I P
(c)

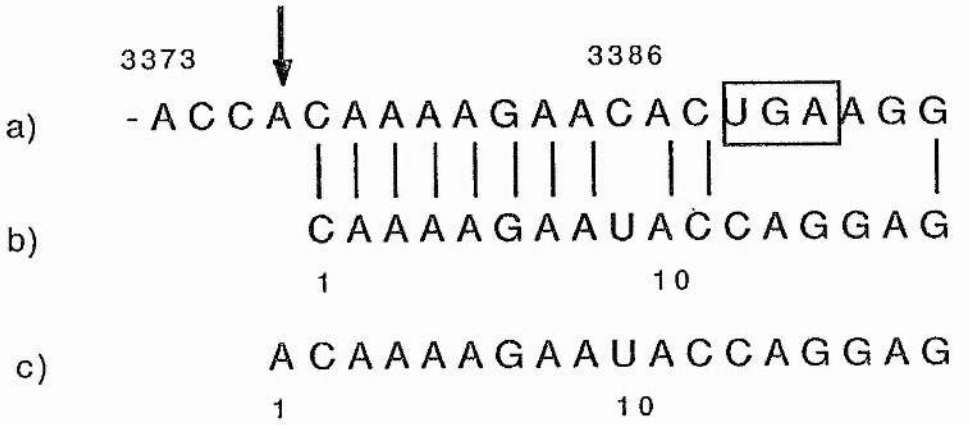
hybridise with the genomic RNA. However, extension from this primer again yielded several products including one which was not present in the sample which was primed on virus particle RNA. The size of this product was approximately 142 nucleotides in length (Fig. 3.2(b)), as determined by comparison with the sequence of M13mp18 (established by dideoxynucleotide chain termination) which was run on the same gel as the primer extension samples. The product was still too large to be accurately sized on the autoradiograph so a third primer (primer 3), complementary to the sequence 179 nucleotides upstream of the coat protein AUG, was used. Extension from this primer yielded a product that comigrated with a dideoxynucleotide-terminated product of 56 nucleotides (Fig. 3.2(c)). From this, it was concluded that the subgenomic RNA starts at nucleotide 3376 (nucleotide 3481 in the sequence of Mayo *et al.*,1989; Miller and Mayo, 1991).

3.5 COMPARISON OF THE 5'ENDS OF THE SUBGENOMIC AND GENOMIC RNAS IN DIFFERENT PLANT VIRUSES

Comparison of the 5'end of the subgenomic RNA of PLRV with that of the genomic RNA reveals the existence of a direct repeat sequence (using the sequences established by van der Wilk *et al.*, 1989, and Keese *et al.*, 1990) between the first 8 or 9 nucleotides and from nucleotide 3376 or 3377

FIGURE 3.3: The sequence similarity found between the 5'-termini of the genomic and subgenomic RNAs of PLRV.

(a) and (b) are sequences from Dutch PLRV (van der Wilk *et al.*, 1989), (c) is sequence from Australian PLRV (Keese *et al.*, 1990). The numbers indicate position relative to the 5'-ends of the RNA. The arrow indicates the first nucleotide of the subgenomic RNA. The termination codon of the putative polymerase gene is indicated by a box.


3373
3386
a)
- A C C A C A A A A G A A C A C U G A A G G
b)
C A A A A G A A U A C C A G G A G
1
10
c)
A C A A A A G A A U A C C A G G A G
1
10

in the genomic sequence (Fig. 3.3). This reinforces the suggestion that the deduced 5'end of the subgenomic RNA of PLRV is correct. Furthermore, there is a similar correspondence between the 5' terminal sequence of the genome and a sequence near the 3' end of the putative polymerase gene in the RNA of BWYV (Veidt *et al.*, 1988). Recent results (V. Ziegler-Graf, personal communication) indicate that this region is where the subgenomic RNA of BWYV starts.

Sequences which are identical to each other at the 5' termini of genomic and subgenomic RNAs have also been observed for several other plant viruses. For example, there is a match of 12 nucleotides in maize chlorotic mottle virus RNA (MCMV; Lommel *et al.*, 1991), up to 10 in tobacco rattle virus (TRV; Cornelissen *et al.*, 1986) and up to 11 in alfalfa mosaic virus RNA (AMV; Symons, 1985). Potential functions for this repeat sequence and putative promoter regions are discussed further in chapter 6.

3.6 THE COMPARISON OF PLRV GENOMIC AND SUBGENOMIC RNA IN PROTOPLASTS FROM SOURCE PLANTS OF DIFFERENT SPECIES

The results described above were mostly obtained with RNA extracted from protoplasts isolated from *N. tabacum* cv. Xanthi. To show that these results were not peculiar to these specific protoplasts and were, in fact, well conserved between various RNA sources in terms of migration and

TABLE 3.1: Plant species and protoplast source plant species, PLRV-infected and healthy, used for RNA extraction.

Letters a-d refer to superscripts on p. 106.

a. Plants transformed with the *Agrobacterium tumefaciens* vector pSCR107 (Barker *et al.*, 1992) which contained the PLRV sequence from nucleotide 3582-4581 (coat protein ORF) under the control of a CaMV 35S promoter.

b. A clone from the progeny of a selfing cross of a cultivar Dr. MacIntosh. Extremely susceptible to PLRV infection.

c. Plants of breeding clones selected from the SCRI breeding programme. Highly resistant to virus multiplication (Barker and Harrison, 1985).

d. Plants transformed with the *Agrobacterium tumefaciens* vector pBIN-CP (Bertioli *et al.*, 1991) which contained the coat protein gene of ArMV.

1. PROTOPLAST SOURCE PLANT SPECIES

Nicotiana tabacum cv. Xanthi

B4 transgenic line of *N. tabacum* cv. Xanthi[d]

N. tabacum cv. Samsun

F4 transgenic line of *N. tabacum* cv. Samsun[a]

N. clevelandii

2. PLANT SPECIES

Physalis floridana

N. tabacum cv. Samsun

F13 transgenic line of *N. tabacum* cv. Samsun[a]

F34 transgenic line of *N. tabacum* cv. Samsun[a]

Solanum tuberosum varieties Maris Piper

Dr. MacIntosh[b]

Katahdin

G74451[c]

B1 and B3 transgenic lines of *S. tuberosum*, variety Desiree[a]

separation of RNA on denaturing gels, a series of blots were made using RNA extracted from different PLRV-infected and healthy plant species, and from PLRV-infected and mock-inoculated protoplasts isolated from different species of host plant. Transgenic plants were also used for RNA analysis, see Table 3.1 for details of the plant and protoplast species used.

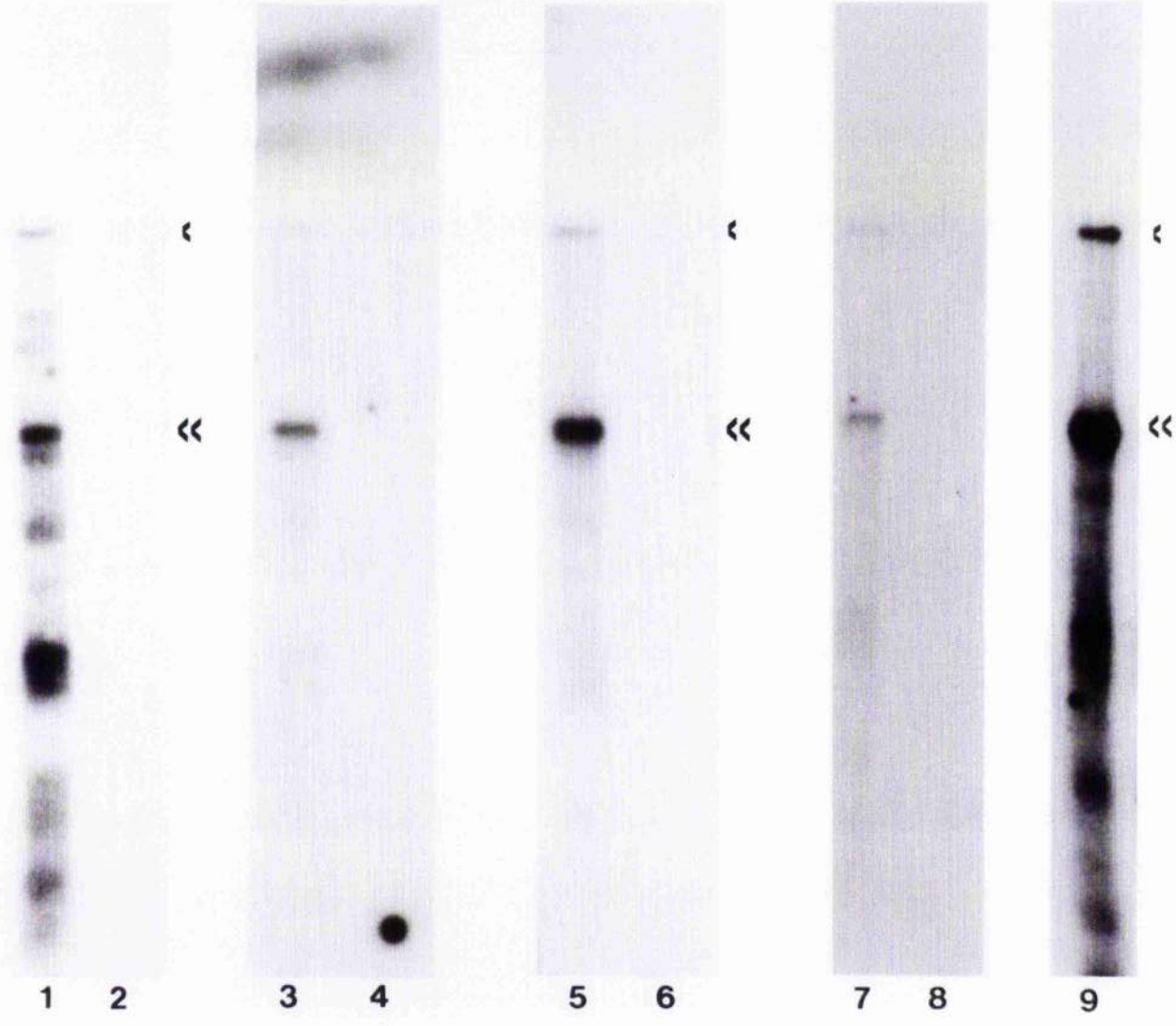
In the northern blots described below, probes A, B and C as detailed in Figure 2.5 were used for hybridisations.

Protoplasts were isolated from *N. clevelandii* and transgenic lines of *N. tabacum* cvs Xanthi and Samsun. Plants of the F4 transgenic line of *N. tabacum* cv Samsun were transformed with *Agrobacterium tumefaciens* containing the binary expression vector pSCR107. This vector contained the PLRV sequence from nucleotides 3582 - 4581 (comprising the coat protein gene and part of the preceeding untranslated sequence) under the control of a cauliflower mosaic virus 35S promoter as according to Barker *et al.* (1992). Plants of the B4 transgenic line of *N. tabacum* cv Xanthi were transformed using *Agrobacterium tumefaciens* containing the vector pBin-CP as described by Bertioli *et al.* (1991). This vector contained the coat protein gene of arabis mosaic virus (ArMV), under the control of a CaMV 35S promoter.

Protoplasts were isolated as described in section 2.2, inoculated with PLRV and, after a period of time in culture, extracted for RNA. After northern blotting and hybridisation with probe A, the resulting tracks containing RNA extracted from PLRV-infected protoplasts showed the PLRV genomic and subgenomic RNAs in their expected positions together with

FIGURE 3.4: RNA extracted from PLRV- and buffer-inoculated protoplasts isolated from different source plants.

RNA was extracted from PLRV- and buffer-inoculated protoplasts which had been isolated from *N. clevelandii* (lanes 1 and 2), *N. tabacum* cv. Xanthi (lanes 3 and 4), the B4 transgenic line of *N. tabacum* cv. Xanthi (transformed with the ArMV CP gene, lanes 5 and 6), *N. tabacum* cv. Samsun (lanes 7 and 8), the F4 transgenic line of *N. tabacum* cv. Samsun (transformed with the PLRV CP gene, lane 9). RNA from PLRV-infected protoplasts is in lanes 1, 3, 5, 7 and 9 and RNA from buffer-inoculated protoplasts is in lanes 2, 4, 6 and 8. Single arrow heads indicate genomic RNA and double arrow heads indicate subgenomic RNA.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8
9

several other, fainter bands (Fig. 3.4) owing to non-specific hybridisation. No hybridisation was seen in the mock-inoculated wildtype or transgenic protoplast tracks (Fig 3.4, lanes 2, 4, 6 and 8). In the protoplasts isolated from the transgenic plants, the PLRV RNA species hybridised strongly and migrated similarly in each track (Fig 3.4, lanes 5 and 9) showing that neither the presence of the PLRV nor the ArMV coat protein gene in the transgenic plant sample appeared to have affected PLRV multiplication or replication. The presence of the PLRV coat protein gene in the plant DNA was not detected since probe A (Fig. 2.5) was used for hybridisation.

It is possible that initial entry of virus particles into cells may be inhibited by the presence of the coat protein of the same virus (Beachy *-et al.*, 1990). In the protoplast system, this does not appear to have happened. This could be due to the artificial circumstances which surround the infection of protoplasts with virus particles. Protoplasts are inoculated under conditions which have been optimised to achieve the highest possible efficiency of infection and these conditions probably overcome any innate resistance to infection which may exist. Furthermore, coat protein is found in only very low quantities in the transformed plants described here (H. Barker, personal communication), inplying that if this is the method of resistance it may be low in this case anyway.

It seems that in protoplasts, neither different cultivars of source plant nor the presence of a gene foreign to the plant and in some instances, the virus, affect the migration of the PLRV RNA. It is not possible to comment on potential inhibitory effects on the multiplication of the virus using the

northern blot data presented here, since in these experiments the northern blots were not designed to be quantitative. However, the presence of the subgenomic RNA on these blots indicates that virus multiplication is taking place.

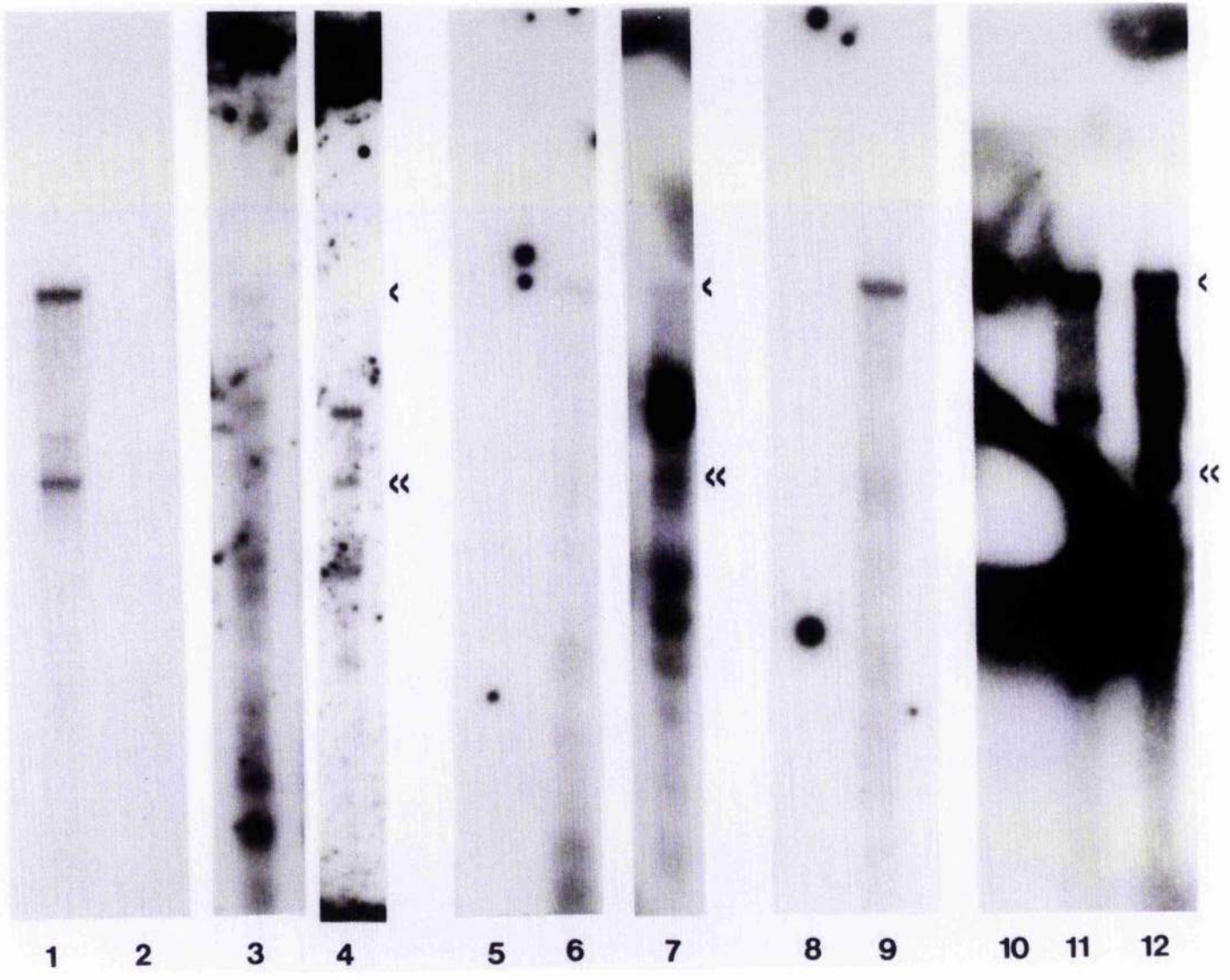
3.7 PLRV RNA SPECIES EXTRACTED FROM DIFFERENT SPECIES OF TRANSFORMED AND NON-TRANSFORMED PLANT

In PLRV-infected plant tissue, the results were very similar to those obtained from PLRV-infected protoplasts. RNA was extracted from PLRV-infected potato varieties Maris Piper, Katahdin, Dr. MacIntosh, G74451 (SCRI breeding clone) and the B1 and B3 transgenic lines of the cv Desiree which were transformed with the same vector as described for the tobacco transformations described in section 3.6, the F13 and F34 transgenic lines of *N. tabacum* cv Samsun transformed as described previously, and *Physalis floridana* (Table 3.1b).

Fig. 3.5 shows RNA extracted from the different plant species described. The RNA extracted from the variety Maris Piper, used for propagating the virus, shows the PLRV RNA to be in positions identical to those found in RNA extracted from infected protoplasts (Fig 3.5, lanes 1 and 2). There was more genomic RNA present than subgenomic and a degree of non-specific hybridisation also took place owing to the greater quantities of plant RNA that were present in these samples than in the protoplast samples.

FIGURE 3.5: RNA extracted from different healthy and PLRV-infected plant species.

RNA was extracted from the leaf tissue of the potato varieties Maris Piper (lanes 1 and 2), Dr. MacIntosh (lane 3), Katahdin (lane 4), G74451 (lanes 5 and 6) and the B1 (lane 7) and B3 (lanes 8 and 9) transgenic lines of the variety Desiree. RNA was also extracted from leaf tissue of *Physalis floridana* (lanes 10-12). Lanes 1, 3, 4, 6, 7 and 9 contain RNA extracted from plants infected with the PLRV Scottish isolate. Tracks 2, 5, 8 and 10 contain RNA extracted from healthy plants. Lanes 11 and 12 contain RNA extracted from *P. floridana* infected with PLRV isolates 11 and 30 respectively. Single arrow heads indicate genomic RNA, double arrow heads indicate subgenomic RNA.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3.7.1 RNA EXTRACTED FROM *PHYSALIS FLORIDANA* AND *N. TABACUM*

The tracks containing RNA extracted from *Physalis floridana* plants infected with PLRV isolates 11 and 30 show a PLRV RNA profile which is in keeping with those already established (Fig. 3.5, lanes 10-12). Reasonably strong non-specific hybridisation has taken place in the PLRV tracks but the healthy sample shows no such reaction.

Northern blots of RNA extracted from wild-type tobacco and plants transformed with the CP gene of PLRV (Barker *et al.*, 1992) did not contain any virus-specific bands. The only bands present were due to non-specific binding and the bands in the healthy tracks were identical in terms of migration distance to those in the PLRV-infected tracks. This implies that PLRV infection of these plants was poor, or that the initial level of infection had dropped sufficiently that multiplication had become undetectable by northern blotting.

The low rate of virus multiplication could be due to the plants having some resistance to virus infection or subsequent multiplication owing to the presence of the virus coat protein gene in the plant genome. Since the plants were originally deemed infected by means of an ELISA test, resistance may have acted to allow only a very low rate of infection or multiplication to occur, the resulting progeny being in too small quantities to be detected by the northern blot. Tobacco plants which have been transformed with the coat protein gene of a virus such as TMV show some resistance to that virus

(Beachy *et al.*, 1990) although the mechanism of resistance is as yet unknown.

However, when protoplasts were isolated from uninfected plants which had been transformed with the PLRV CP gene construct described previously (section 3.6, Barker *et al.*, 1992), they appeared to be susceptible to PLRV infection and allowed subsequent virus multiplication to proceed. This suggests that, in this case, some of the resistance shown by the transgenic plant may be involved in the initial infection process. This process is almost completely disrupted in the infection of protoplasts when the major objective is to ensure that the virus particles infect the protoplasts. The protoplast isolation procedure combined with this rigorous infection process may mean that resistance mechanisms are bypassed and so are ineffective. However, it has been shown that protoplasts isolated from plants transformed with the TMV coat protein gene were protected against infection with TMV (Register and Beachy, 1988).

3.7.2 RNA EXTRACTED FROM POTATO

Of the several potato cultivars which were used for RNA extraction, the non-transformed cultivars "Dr. MacIntosh" and "Katahdin" showed similar profiles on northern blots in that the hybridisation with the viral RNA was very weak compared to the hybridisation with the plant ribosomal RNA (Fig. 3.5, lanes 3 and 4 respectively). The weak hybridisation which was

apparent in the Katahdin sample, however, shows comparable migration to that achieved for PLRV-infected Maris Piper RNA (Fig. 3.5, lanes 1 and 2) although the subgenomic RNA band was much stronger than the genomic band. In comparison, the subgenomic RNA in the Dr. MacIntosh sample was similar in strength to the genomic RNA, both being rather faint. This may suggest that more virus replication was taking place in the Katahdin variety although the Dr. MacIntosh cultivar is known to be very susceptible to PLRV infection (H. Barker, personal communication).

In samples of total RNA extracted from the transgenic lines B1 and B3 of the potato cultivar Desiree, transformed with the vector as described in section 3.6 (Barker *et al.*, 1992), plant ribosomal RNA also hybridised more strongly, in some cases, to the probe used than did viral RNA. RNA extracted from PLRV-infected B1 plants showed the subgenomic RNA band to be slightly darker than the genomic RNA band (Fig. 3.5, lane 7). RNA extracted from PLRV-infected B3 plants contained only the genomic band on northern blots (Fig. 3.5, lanes 8 and 9). This complete absence of subgenomic RNA could indicate that there was no virus multiplication taking place at the time of the RNA extraction. However, the plant tissue was infected since there is clear evidence of the presence of the genomic RNA. This RNA sample was extracted from a plant which was grown up from a PLRV-infected tuber so it is possible that the virus can spread effectively through the plant from the infected tuber but is not allowed to replicate because of the presence of the virus coat protein which appears to generate resistance of some kind. This is probably a more realistic test for virus replication in

transgenics than the infection of protoplasts isolated from transgenic plants, since there is no need for elaborate infection procedures.

This RNA pattern was also observed for RNA extracted from potato plants which were known to be extremely resistant to PLRV infection (Fig. 3.5, lanes 5 and 6). These plants, designated G74451, were derived from a SCRI breeding programme, and as for all the plants described in these results, the tissue used for RNA extractions was taken from plants which had been grown up from infected tubers. Since it is known that this variety is resistant to infection, as are the B3 plants, the same conclusion must be reached, that the virus can move through the plant in the phloem tissue but is not able to replicate in large quantities, at least not in the leaf tissue.

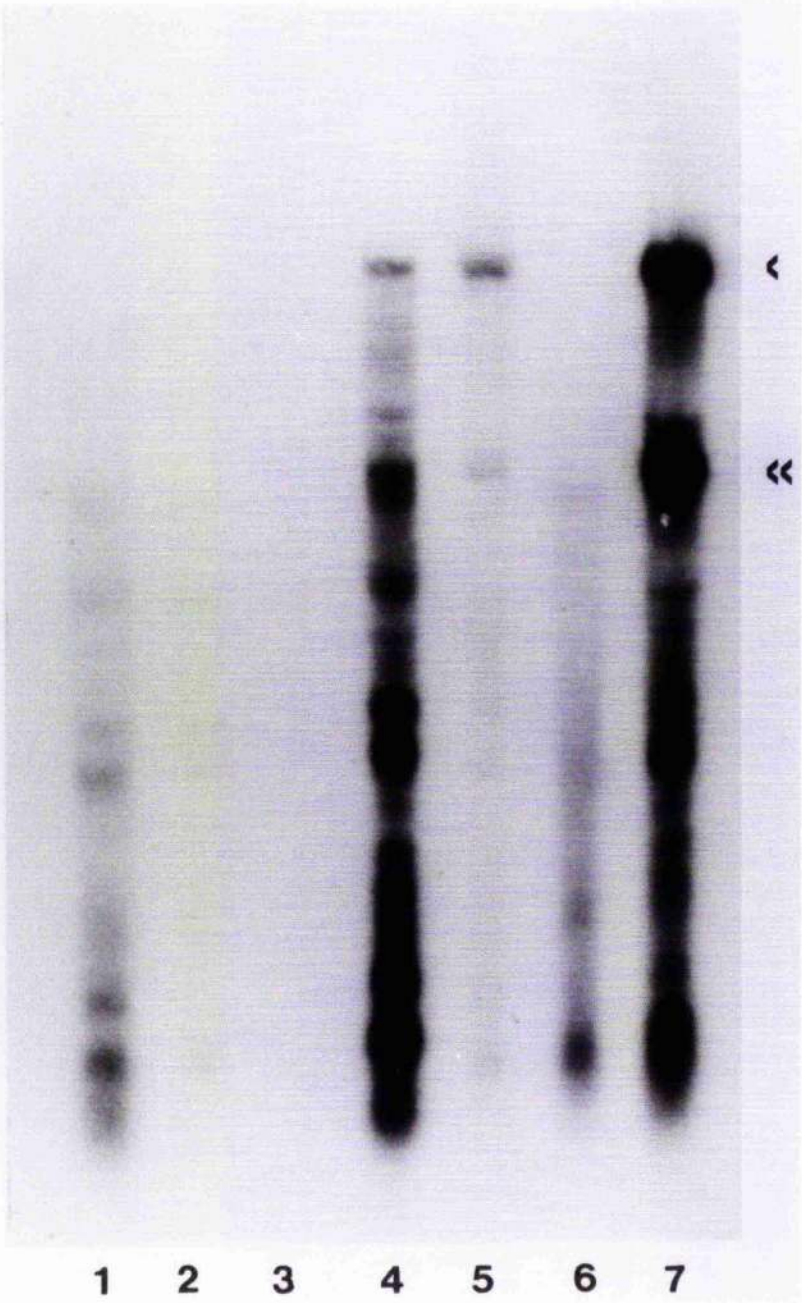
3.8 PLRV RNA EXTRACTED FROM DIFFERENT POTATO TISSUES

RNA was extracted from different tissues of the potato variety Maris Piper, healthy and PLRV-infected, 26 and 72 days after planting. The tissues used for RNA extraction were the leaves, petiole, stem and root. RNA was also extracted from healthy and PLRV-infected tubers which had been stored at 4°C for 16 and 76 days after harvesting and stolons derived from the tubers which had been stored for 76 days.

The RNA which was extracted from the youngest samples, i.e. the 26 day tissue samples and the 16 day tuber samples were in the best condition.

FIGURE 3.6: RNA extracted from different tissues of a healthy and PLRV-infected potato plant.

RNA was extracted from healthy and PLRV-infected potato tissue 26 days after planting, and healthy and PLRV-infected tubers, after 16 and 76 days of dark storage at 4°C. Lane 1 contains RNA from PLRV-infected tubers after 16 days of storage, lane 2 contains RNA from a tuber after 76 days of storage, lanes 4-7 contain RNA extracted from the leaf, petiole, root and stem tissue, respectively, of a 26 day old plant. All lanes contain RNA extracted from PLRV-infected tissue, except for lane 3 which contains RNA extracted from a healthy tuber. Tracks containing RNA extracted from PLRV-infected stolon tissue contained no bands (not shown). A single arrow head indicates genomic RNA and a double arrow head indicates subgenomic RNA.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WU, J.G., LU, W.J., TIEN, B. & QUI, B.S. (1985). Multiplication of velvet tobacco mottle virus encapsidated virusoid RNA in *N. clevelandii* protoplasts. *Acta Microbiologica Sinica*, **25**, 221-226.

YAMAOKA, N., FURUSAWA, I. & YAMAMOTO, M. (1982). Infection of turnip protoplasts with cauliflower mosaic virus DNA. *Virology*, **122**, 503-505.

YEH, S.D. & CHEN, Y.F. (1988). Replication of papaya ringspot virus in susceptible and resistant lines of Cucumis metuliferus. *Plant Protection Bulletin, Taiwan*, **30**, 269-278.

ZELCER, A., WEABER, K.F. BALAZS, E. & ZAITLIN, M. (1981). The detection and characteristics of viral-related double-stranded RNAs in tobacco mosaic virus-infected plants. *Virology*, **113**, 417-427.

ZHENG, Y. & EDWARDS, M.C. (1990). Expression of resistance to barley stripe mosaic virus in barley and oat protoplasts. *Journal of General Virology*, **71**, 1865-1868.

Of the tuber samples, there was no reaction with the RNA extracted from the healthy samples and only the PLRV-infected sample which had been stored at 4°C for 16 days after harvesting appeared to contain PLRV-specific RNA, although the sample which had been stored for 76 days showed a small amount of non-specific hybridisation (Fig. 3.6, lanes 1-3). Additionally, no virus-specific RNA was detected in RNA extracted from the stolons which had been present on the older tubers.

In the RNA extracted from the tuber which had been cold-stored for 16 days, there was a faint band which corresponded to PLRV genomic RNA only. This implies that the tuber is infected and contains virus particles but that active virus multiplication is not taking place at this time. It is possible that the virus is unable to multiply in the tuber due to unsuitable conditions and that the particles are merely stored until they can move into the phloem tissue. Moreover, it may be that as the tuber is stored at 4°C for longer periods of time, a proportion of the particles are destroyed through their own instability, bad conditions in the tuber or potential defence systems which may exist, leaving enough to continue the infection but too few to be detected on a northern blot.

Of the RNA samples which were extracted from the different tissues of a potato plant, the young tissue gave the best RNA and that extracted from the older tissue appeared to be rather badly degraded.

The RNA which was extracted from the stem tissue appeared to contain the most PLRV RNA per 5µg of total RNA used for gel electrophoresis (Fig. 3.6, lane 7). The RNA extracted from the leaf tissue

contained the second highest quantity of PLRV RNA. There did not appear to be any PLRV-specific RNA present in the root tissue, although a very faint shadow was present at the position of the genomic RNA. It is possible that if more RNA was loaded initially, a genomic band would be present (Fig. 3.6, lanes 4-6).

These results show that most of the PLRV replication is taking place in the stem of the plant where the major phloem vessels are located. Multiplication is also taking place in the leaves and petioles of the plant, but appears not to be taking place in the roots or tubers.

The older tissue may contain degraded RNA owing to the physiological condition of the plant itself. Because of virus infection, the plant deteriorates quickly and the nutrients needed by cells to effect virus production may no longer be available, due to the impaired phloem transport system. This may mean that due to prolonged exposure to the destructive influences of the virus, cells in which virus RNA is normally found intact have been damaged and the virus RNA itself degraded. As this takes place, virus replication and multiplication machinery become impaired and the virus can no longer multiply.

Furthermore, it may be that virus multiplication stops as the plant reaches a certain age because of internal conditions associated with ageing. At this point particles are deposited in the tuber during tuber formation. This may also account for the poor condition of RNA and particles in other areas of the plant.

3.9 NON-SPECIFIC RNA HYBRIDISATION

Through the course of the northern blots described here, non-specific hybridisation has been a common occurence. Seen more commonly in RNA samples taken from plants than protoplasts, although occasionally found in both, it probably occurs mainly because of plant RNA being present in vastly greater quantities than virus RNA in a total RNA sample.

When gels were stained with ethidium bromide before blotting, these bands were also visible indicating that they are not present solely because of virus RNA. In these samples viral RNA is present in quantities too small to be visualised directly on a stained gel. Major bands present on the blots generally corresponded to ribosomal RNA, but other RNA bands corresponding to smaller RNA molecules were also observed. These bands tend not to be visible in the healthy tracks, suggesting that they are virus-associated. It is possible that these are caused by the hybridisation of fragmented pieces of RNA, derived from both viral and plant origins. Since they contain virus RNA, they subsequently hybridise with the specific probe. Smith and Harris (1990) also noticed minor hybridisable RNAs but suggested that these were probably partial degradation products of the two major hybridisable RNAs "stacking" with plant ribosomal RNA during electrophoresis.

Furthermore, it is possible that the probe used to detect the presence of virus RNA would also detect plant ribosomal RNA due to the chance occurrence of similar sequences. However, in most cases described here, the

control, healthy RNA sample was negative implying that it was a combination of the plant and virus sequences which were reponsible for the non-specific hybridisation.

3.10 DISCUSSION

The results described here show that the subgenomic RNA of PLRV is 2505 nucleotides in length, starting at position 3376 in the sequence of van der Wilk *et al.* (1989) with a 5'-untranslated leader sequence of 212 nucleotides. A direct repeat sequence of 8 nucleotides is present at the 5'-ends of the genomic and subgenomic RNAs which suggests that the extreme 5'-ends are functional, possibly as recognition sites for the replicase complex in the negative-strand RNA. They may also have a function in the translation of the ORFs.

Attempts to establish the presence or absence of a genome linked protein (VPg) on the subgenomic RNA were unsuccessful (not shown). A VPg is known to be located at the 5'-end of the genomic RNA (Mayo *et al.*, 1982b) and for some other viruses, it has been proposed to be also located at the 5'-end of the subgenomic RNA, e.g. southern bean mosaic virus (SBMV, Ghosh *et al.*, 1981). The function of the VPg is unknown but it is thought that it may act as primer for RNA synthesis and there is a possibility that it may protect the RNA to some extent from degradation by intracellular

ribonucleases. If these functions were fulfilled, it would be desirable for the virus to have a VPg at the 5'-end of the subgenomic RNA.

The proportion of genomic to subgenomic RNA seems to vary between the different samples examined. This probably reflects differences between protoplasts and plants in terms of virus multiplication, i.e. differences in quantities of virus, timing of multiplication and preferred conditions of virus multiplication. The presence of the subgenomic RNA indicates that virus multiplication was taking, or had taken place in the cell. The results appear to have shown that PLRV spreads throughout the whole plant but does not necessarily multiply in every tissue. It is also evident that in plants such as the B3 transgenic line and the breeding clone G74451, which have been shown to have resistance to PLRV infection, virus spread is not restricted but multiplication is. These plants were grown up from infected tubers, so virus spread may be more thorough than if the infection was via aphid transmission. Protoplasts do not appear to show the same levels of resistance to virus multiplication that whole plants are capable of.

These results imply that resistance to PLRV, in secondary infections at least, inhibits virus multiplication in the cell rather than initial infection or cell to cell spread. Obviously, in the transgenic plants, resistance is connected with the presence of the viral gene which may be expressing the virus coat protein. Whether this affects the uncoating of the virus or subsequent RNA transcription is unknown but it appears that something similar takes place in the cells of the resistant breeding clone, G74451.

This result outlines a problem in the detection of this type of resistance. It suggests that if resistant plants can be infected but resist virus multiplication and show only slight symptoms, spread from plant to plant and therefore crop to crop is still possible, although less likely than for a susceptible crop. This may allow for the spread of the virus from one resistant potato crop to a susceptible one.

When RNA was extracted from different parts of a potato plant, differences in transcription of viral RNA were evident between the different tissues. Most appeared to take place in the stem of the plant where the major phloem vessels are and there appeared to be no PLRV-specific RNA present in extracts of root or tuber tissue. The apparent absence of PLRV-specific RNA in the tuber may be deceptive, since there may only be a very small quantity of virus present in this tissue. Otherwise the virus seems able to move into the phloem in most tissues of the plant.

These results seem to imply that the virus cannot or does not replicate in all plant tissues, further testing for this could be done by *in situ* hybridisation or by sectioning followed by immunogold labelling or tissue printing. It is unknown whether this is due to some kind of resistance to uncoating or replication or simply due to the surrounding conditions being inappropriate for these processes. Virus multiplication is denoted on northern blots by the presence of the subgenomic RNA. This perhaps shows the importance of northern blots in the determination of virus infection. Serological tests can quantify accurately the amount of virus which is

present, but without performing several assays over a period of time, they cannot determine if it is actively multiplying in the plant.

Comparison of PLRV RNA extracted from plants and protoplasts of different species showed that only one subgenomic species was present and that both RNA species migrated to the same relative positions with each RNA extract. This result was the same when different PLRV isolates were used.

4. THE MULTIPLICATION OF PLRV IN TOBACCO PROTOPLASTS

4.1 INFECTION OF PROTOPLASTS BY PLRV

In chapter 3, it has already been established that PLRV can infect tobacco mesophyll protoplasts and multiply therein. This chapter attempts to describe and discuss PLRV multiplication in this system and define conditions resulting in improved virus yield.

Although the plants used mostly for protoplast isolation were *N. tabacum* cv. Xanthi, PLRV was also shown to infect protoplasts isolated from the leaves of *N. tabacum* cv. Samsun (Fig. 3.4), *Chenopodium quinoa* and *N. clevelandii* (Fig 3.4). Infection of *C. quinoa* was detected by ELISA only, the poor survival rate of the protoplasts meant that RNA and protein extractions were not possible.

Protoplasts isolated from Xanthi tobacco plants were used to investigate the multiplication of PLRV. Initially, the inoculation conditions of the system were examined to determine points which could be optimised. The multiplication of the virus in protoplasts was examined using time course experiments and the effects of different light conditions on virus replication were observed.

4.2 OPTIMISATION OF CONDITIONS OF INFECTION OF PROTOPLASTS BY PLRV

The different components of the inoculation mixture have already been discussed in Chapter 1. The concentration of virus which gave the best infection was initially reported by Kubo and Takanami, (1979), to be 1 μg/ml. Experiments were done to establish the optimal concentration of PLRV virus particles needed for protoplast infection.

4.2.1 THE EFFECT OF VIRUS INOCULUM CONCENTRATION ON SUBSEQUENT VIRUS MULTIPLICATION IN PROTOPLASTS

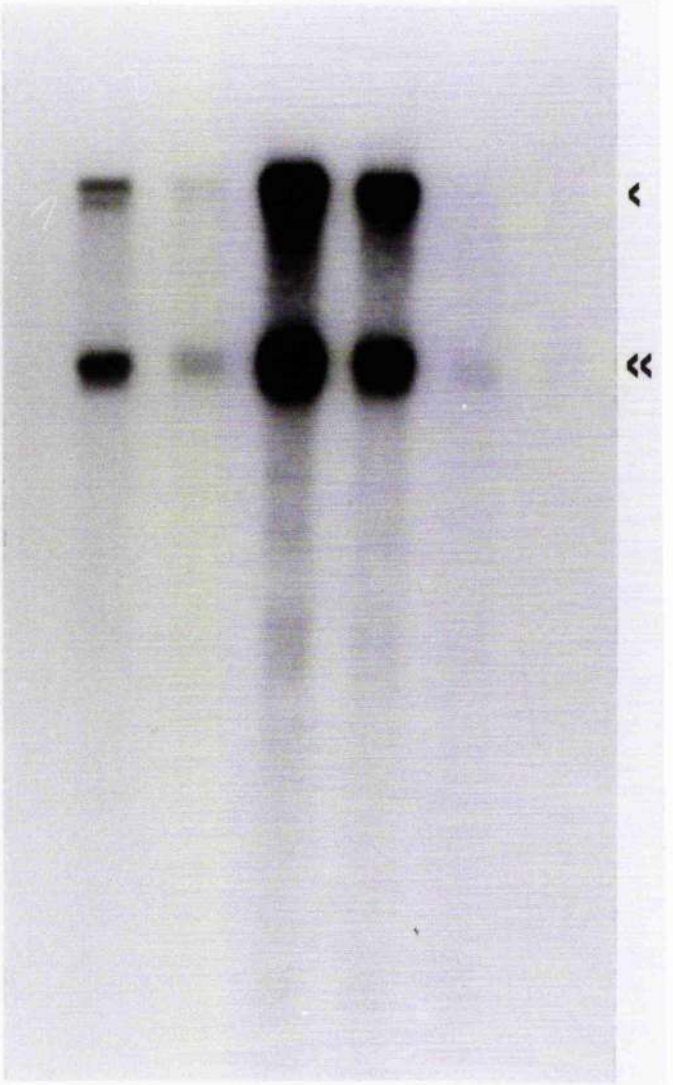
To establish the optimal concentration of infection of a freshly made preparation of PLRV virus particles, protoplasts were inoculated in the usual way but with a range of concentrations of PLRV. These were 0.4, 0.2, 0.1, 0.05, 0.025 and 0.0125 μg/ml (final concentration), each in a volume of 5μl.

The samples were harvested after 48 hrs of incubation, part of each was used for an ELISA assay and part for an RNA extraction. Northern blot analysis showed that most virus RNA was present in the extract of protoplasts inoculated with 0.1 μg/ml PLRV (Fig. 4.1).

This result was corroborated by the ELISA assay and the percentage infection assay. The ELISA test confirmed that most virus protein was present in the sample inoculated with 0.1 μg/μl PLRV and the percentage

FIGURE 4.1: Northern blot of RNA extracted from cultured protoplasts after inoculation with different concentrations of PLRV.

RNA was extracted from protoplasts which had been inoculated with concentrations of 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125 µg/ml PLRV virus particles and subjected to northern blotting (samples are in lanes 1-6 respectively). A single arrow head indicates the genomic RNA and a double arrow head indicates subgenomic RNA.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6

infection results show that this sample contained the highest number of infected protoplasts.

When a higher concentration of virus particles was used, a lower amount of infection was achieved. This occurs because higher concentrations of virus require higher concentrations of PLO to promote infection. However, PLO must be used at a concentration which allows infection of the protoplasts but which is not so concentrated as to damage them irrepairably (Sander and Mertes, 1984). Therefore increasing the amount of PLO used is counter-productive and consequently limits the amount of virus inoculum which may be used.

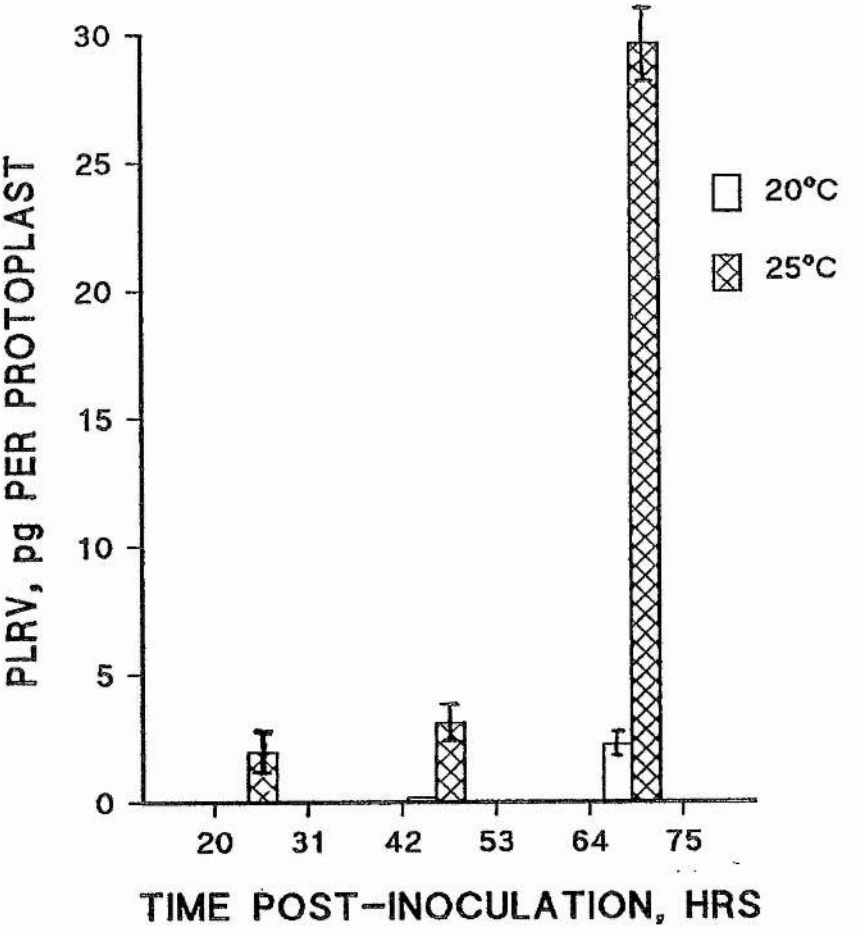
4.2.2 THE EFFECT OF INCUBATION TEMPERATURE ON VIRUS MULTIPLICATION IN PROTOPLASTS

Another attempt to optimise the infection of protoplasts was to test various conditions of culture. As described in Chapter 1, the culture of protoplasts generally takes place at about 20-25°C in constant light (Kubo *et al.*, 1975b), although incubation has been reported at temperatures as low as 11°C (Barnett *et al.*, 1981) and as high as 30°C (Bajet and Goodman, 1981).

An experiment was done comparing PLRV multiplication in protoplasts which were incubated at 20°C with some which were incubated at 25°C. The protoplasts were harvested at intervals and the amount of virus present was quantified by ELISA. RNA was also extracted from each

FIGURE 4.2a: The effect of temperature on PLRV multiplication in protoplasts.

Protoplasts were inoculated with PLRV virus particles and incubated simultaneously at 20°C and 25°C. Virus concentration in protoplasts was determined by ELISA. The results of 3 separate experiments were used to determine the standard error of the mean (SEM) where possible, this is shown as error bars on the graph (in some instances the SEM was too small to be drawn).


30
25
20
15
10
5
0
PLRV, pg PER PROTOPLAST
20°C
25°C
20
31
42
53
64
75
TIME POST-INOCULATION, HRS

sample.

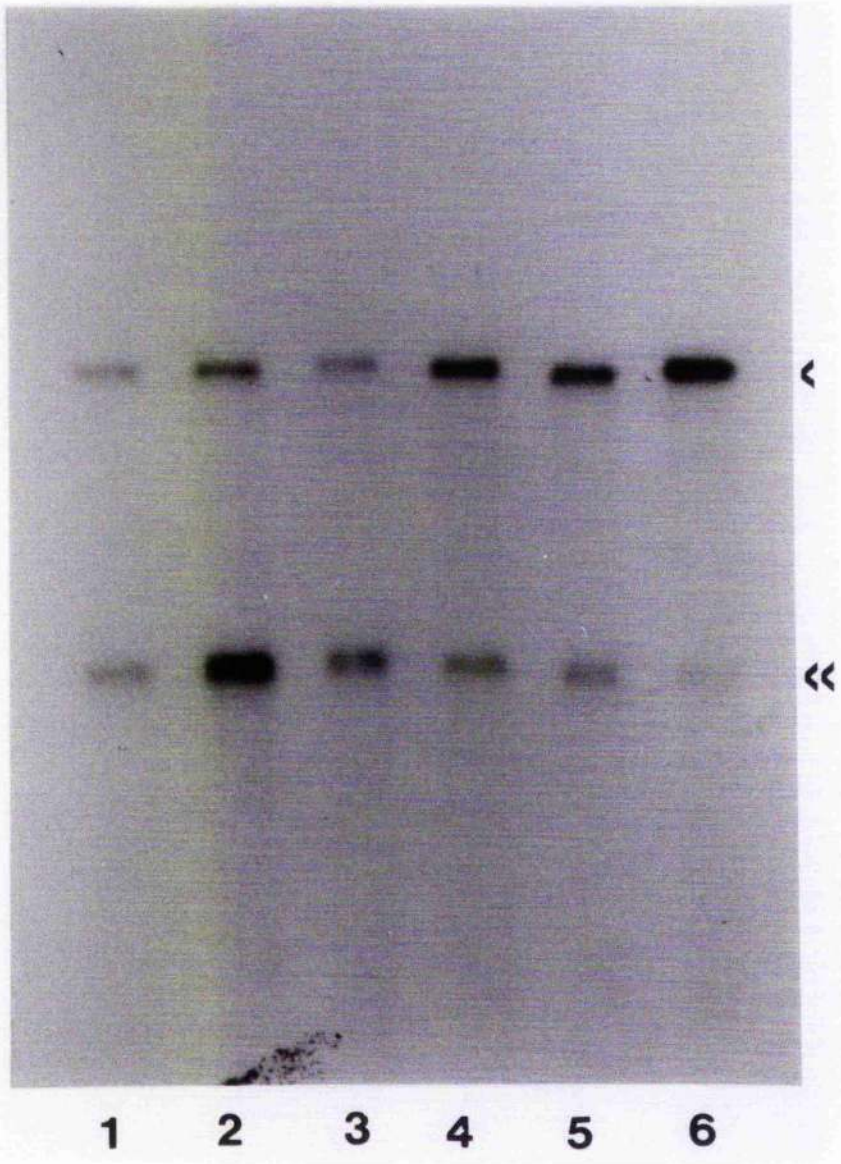
Fig. 4.2a shows that incubation at 25°C appears to result in a dramatic increase in virus multiplication. The northern blot also shows more virus RNA, in particular genomic RNA, present in the samples incubated at 25°C (Fig. 4.2b, lanes 2, 4 and 6).

Although there appears to be more virus multiplication having taken place at 25°C, the protoplasts themselves survive longer and in better condition when incubated at the lower temperature. This effect has also been reported by Takebe (1977). The percentage survival may not differ greatly and the difference is minimal after short periods of culture, but in general, fewer protoplasts survive after 3 days of incubation at 25°C compared to 20°C.

However, although they survive less well, the protoplasts may function better at the higher temperature because of the growth conditions of the source plants. The plants were grown at 25°C for 16 hrs of light and at 20°C for 8 hrs of darkness. The 25°C incubation in light may precondition the protoplasts to be more efficient at this temperature. However, because they are isolated from the rest of the plant, they are less resilient to the stress that higher temperatures may cause and the higher levels of virus production which these promote.

FIGURE 4.2b: Northern blot of RNA extracted from protoplasts which had been incubated at two different temperatures.

RNA was extracted (and northern blotted) from PLRV-infected protoplasts which had been harvested after 24, 48 and 72 hrs of incubation (lane 1 and 2, 3 and 4, 5 and 6 respectively). The RNA in lanes 1, 3 and 5 was extracted from protoplasts which had been incubated at 20°C and the RNA in lanes 2, 4 and 6 was extracted from protoplasts which had been incubated at 25°C. Tracks containing RNA extracted from buffer-inoculated protoplasts contained no bands (not shown). A single arrow head indicates the genomic RNA and a double arrow head indicates the subgenomic RNA.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4.3 PLRV MULTIPLICATION IN TOBACCO PROTOPLASTS; GROWTH CURVE

Time course experiments were conducted where PLRV and mock infected-protoplast samples were harvested at certain times between about 15 and 100 hrs post-inoculation. From these samples, RNA and protein were extracted, the amount of virus present was quantified by ELISA and the percentage of the protoplast sample which had become infected was established. By combining the data obtained from these different protoplast extracts, more details of the timing of virus multiplication in the protoplasts were obtained.

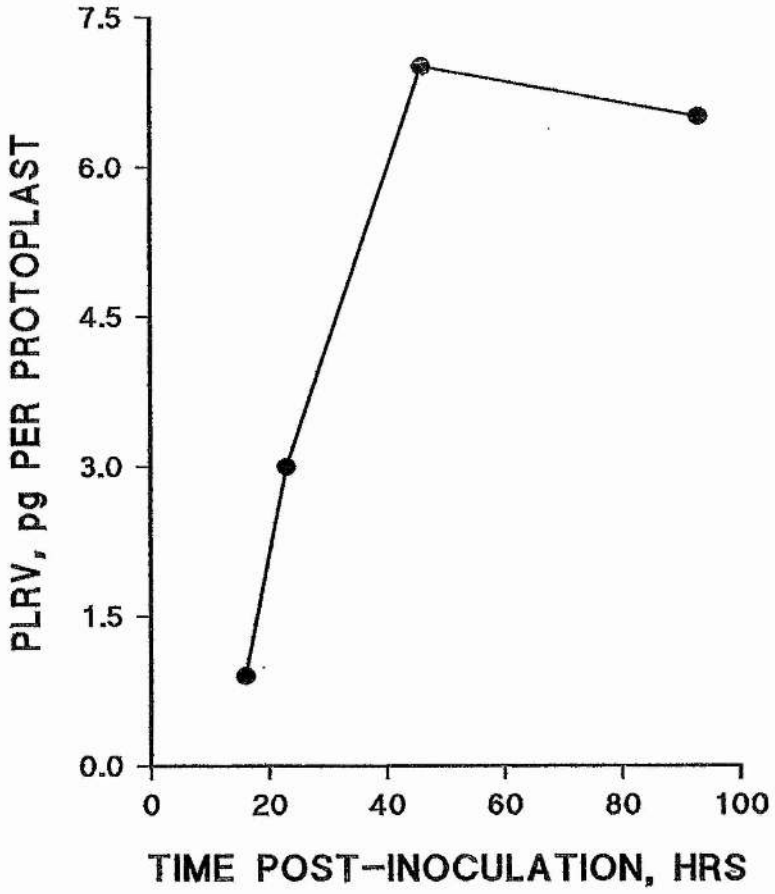
To establish a growth curve for PLRV multiplication in protoplasts, the amount of virus in samples of 1 x 10^6 protoplasts was determined by ELISA.

The growth curve is presented here in terms of PLRV concentration per protoplast (pg), irrespective of infection (Fig 4.3a). The curve shows that up to 24 hrs post-inoculation, there is little infection apparent. The amount of virus present increases linearly until between 40 and 50 hrs post-inoculation and then appears to straighten off with no additional virus accumulation occurring. A similar growth curve in protoplasts has been described for TMV (Huber *et al.*, 1977).

In some protoplast time course experiments, the initial phase where virus particle accumulation was very slow was extended for much longer than the usual 24 hrs and virus multiplication proceeded at a constant rate for over

FIGURE 4.3a: The growth curve of PLRV multiplication in protoplasts.

Protoplasts were inoculated with PLRV, incubated at 25°C and harvested at various times post-inoculation. The virus concentration in the protoplasts was determined by ELISA.


7.5
6.0
4.5
3.0
1.5
0.0
PLRV, pg PER PROTOPLAST
0
20
40
60
80
100
TIME POST-INOCULATION, HRS

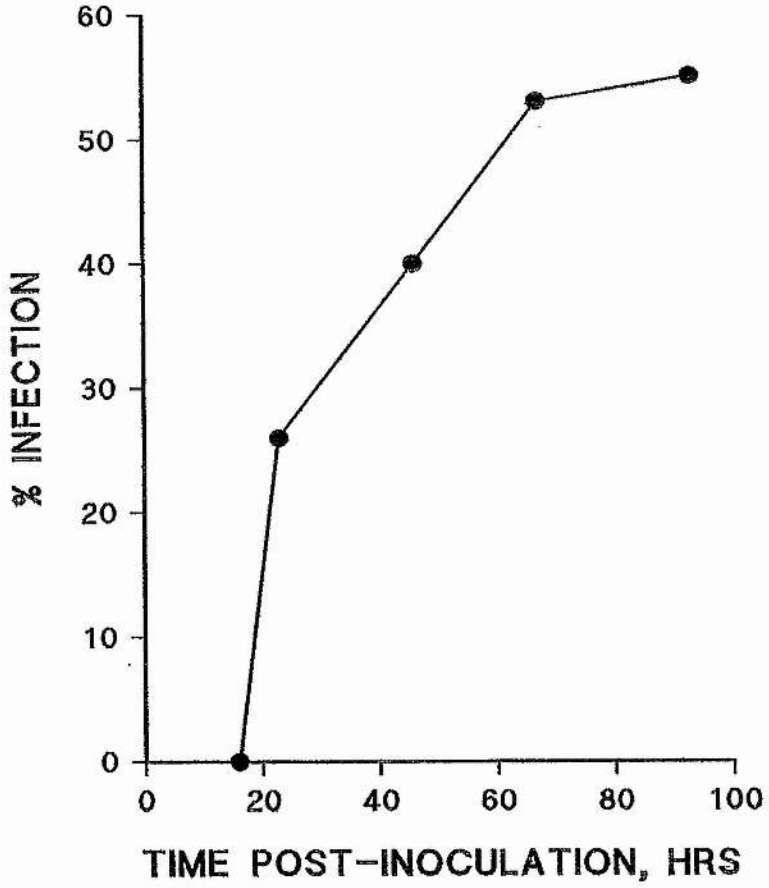
70 hrs post-inoculation. This has been reported to happen in cowpea mosaic virus (CPMV) infection of tobacco protoplasts, and the delay is thought to be due to the difficulty which the virus has in multiplying in plant tissue other than that of its natural host (Huber *et al.*, 1977). This could explain the slightly differing growth curves obtained for different protoplast inoculations with PLRV, but other explanations such as differing physiological conditions of the source plants, and therefore the protoplasts, or only a low rate of infection could also be feasible reasons for delayed virus multiplication.

4.3.1 PERCENTAGE INFECTION OF PROTOPLASTS WITH PLRV

The proportion of cells which had become infected was determined and the representative curve shows a similar trend to that of the growth curve (Fig 4.3b). Only a small number of protoplasts were infected at 24 hrs post-inoculation but this proportion increased until about 50 hrs when the curve levelled off. This suggests that in the majority of cells which are infected, whole virus particles are not synthesised until between 20 and 24 hrs post-inoculation and most of the infected cells contain whole virus particles at about 48 hrs post-inoculation. Since infection is synchronous, the total number of infected protoplasts cannot increase, as the quantity of internal virus does. This implies that it takes about 24 hrs after the inoculation of protoplasts for the virus to produce new particles which can be detected by FITC-labelled antibodies.

FIGURE 4.3b: The curve of percentage infection of protoplasts by PLRV.

Protoplasts were inoculated with PLRV, incubated at 25°C and harvested at various time intervals post-inoculation. The percentage infection of protoplasts was determined by staining protoplasts with FITC-labelled antibodies followed by viewing using a fluorescence microscope.

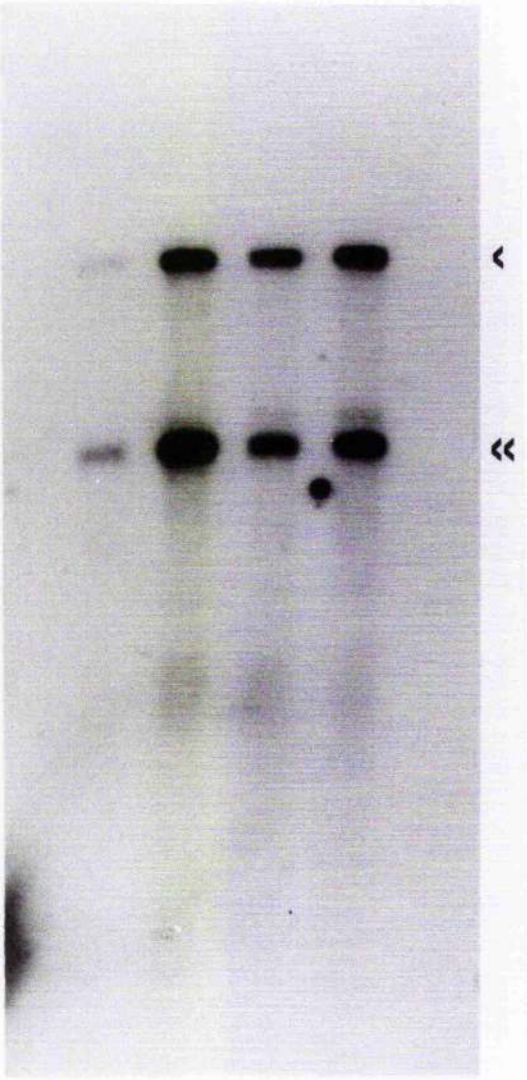

60
50
40
30
20
10
0
% INFECTION
0
20
40
60
80
100
TIME POST-INOCULATION, HRS

4.3.2 RNA EXTRACTED FROM PLRV-INFECTED PROTOPLAST TIME COURSE SAMPLES

The RNA which was extracted from the protoplast samples was northern blotted and hybridised with the probes described in Fig. 2.5. Both RNA species appeared as early as 15 hrs post-inoculation and were present for as long as the time course proceeded, approximately 100 hrs post-inoculation (Fig. 4.3c). A further northern blot was made using RNA extracted from protoplast samples which had been harvested as early as 3.5 and 7 hrs post-inoculation. In these tracks neither the genomic nor the subgenomic RNA of PLRV was present. This suggests that at these times too little virus RNA replication had taken place to be detected on a northern blot. Replication of luteoviruses is thought to proceed with the positive strand being copied into a negative strand which is then used as a template for further positive strand synthesis (Davies and Hull, 1982). In viruses such as those in the tobamovirus and tombusvirus groups replicative intermediates which comprise fully or partially double-stranded molecules have been described in infected cells (Henriques and Morris, 1979; Zelcer *et al*, 1981). These are implicated in the replication of the viral RNA. Therefore, it is possible that in the first few hours after inoculation of protoplasts, before virus replication begins, the virus particles are being uncoated and the RNA is undergoing the replicative processes, such as the synthesis of a negative strand, which are necessary. The virus begins to make RNA in large quantities to be used for translation of proteins. Quantities of RNA large

FIGURE 4.3c: Northern blot of RNA extracted from time-course samples of PLRV-infected protoplasts.

RNA was extracted from PLRV-infected protoplasts 15, 23, 43, 68 and 88 hours post-inoculation (lanes 1-5 respectively), and from buffer-inoculated protoplasts 48 hours post-inoculation (lane 6) and northern blotted. The protoplasts had been incubated at 25°C in continuous light. A single arrow head indicates the genomic RNA and a double arrow head indicates the subgenomic RNA.


1 2 3 4 5 6

enough for detection were present at about 15 hrs post-inoculation.

In the northern blot, the two RNA bands are relatively faint until after 24 hrs post-inoculation, but until this point, there is more subgenomic than genomic RNA present.

The track on the blot which corresponds to the sample taken at 48 hrs is generally the darkest, suggesting that the quantity of viral RNA present reaches a plateau at this point. There is again more subgenomic than genomic RNA present. The next samples at about 68 and 88 hrs post-inoculation contain roughly equal quantities of the two RNAs.

The northern blots of RNA extracted from protoplast time course samples (Fig. 4.3c) echo the growth curve which has already been established (Fig. 4.3a). There is little RNA present before 15 hrs post-inoculation, as there are few virus particles present. The amount of RNA appears to increase until about 48 hrs and then the amount may decrease slightly or remain constant for the rest of the time course. This pattern is very similar to that shown in the growth curve.

The blots also show that RNA replication is an ongoing process in protoplasts. They imply that both species of RNA are constantly being made, the genomic to be packaged into particles and the subgenomic to provide coat protein in order to pack the genomic RNA.

However, it was mentioned earlier that after about 50 hrs of incubation, the amount of virus found in the cells tends not to increase, implying that additional virus particles are not being made, probably because of the protoplasts being no longer able to support such levels of virus

multiplication. Nevertheless, if virus multiplication was not taking place, subgenomic RNA would not be found in protoplast extracts. This suggests that one of two events is taking place:

i. RNA is being stored in the cytoplasm of the protoplasts. This is known to happen in the cytoplasm of plant cells infected with BYDV where fibrillar structures are formed during the accumulation of RNA (Gill and Chong, 1976). It has not been investigated in protoplasts.

ii. There is a constant low level of replication. This would involve virus particles which had been made in the protoplast being uncoated and going through the stages of replication and translation. As mentioned above, the protoplasts, by this time, are in poor condition and are probably incapable of supporting anything more than a low level of replication.

4.3.3 PROTEINS EXTRACTED FROM PLRV-INFECTED PROTOPLAST TIME COURSE SAMPLES

Protein extracts which were made from protoplast samples showed a similar pattern to the RNA and ELISA results. Early samples, up to 24 hrs post-inoculation did not contain large enough quantities of PLRV coat protein to be detected on a western blot immunoblotted with a monoclonal antibody, SCR-2, directed against the coat protein of the virus. Samples taken between 24 and 72 hrs post-inoculation contained apparently increasing quantities of this protein (Fig. 4.3d). Presumably, a sample taken after 72 hrs post-

FIGURE 4.3d: Western blot of protein extracted from PLRV-infected protoplasts.

Protein was extracted from PLRV-infected protoplasts 24, 48 and 72 hrs post-inoculation (lanes 1, 2 and 3 respectively) and from PLRV virus particles (lane 4) and western blotted. A monoclonal antibody (SCR-2) directed against the coat protein of the virus was used for detection purposes. A single arrow head indicates the coat protein and a double arrow head indicates the readthrough protein (P5) of Mr 53K found in virus particles. The Mr of viral proteins were calculated using Sigma protein molecular weight markers (Fig. 2.3)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‹
1
2
3
4

inoculation would contain a similar quantity of protein to that in the 72 hr sample since the ELISA assays show the virus concentration to be relatively static after about 48 hrs of culture.

The readthrough protein was not detected in the protein samples extracted from PLRV-infected protoplasts. This is surprising because Bahner *et al.* (1990) detected readthrough protein in extracts of PLRV-infected protoplasts which had been incubated in near-identical conditions to those described here and identical extraction procedures were used. It is likely that only a few molecules of readthrough protein are present in each virus particle compared to coat protein and therefore may be difficult to detect. However, since Bahner *et al.* (1990) detected it in the same quantity of protoplasts as used here, this may not be the reason as to why it could not be located on a western blot. It may be particularly sensitive to proteases and therefore require protection from these during the extraction procedure.

The environmental conditions surrounding the protoplasts during the incubation period may be very important in the production of the readthrough protein. Slight changes in these conditions may result in different levels of its manufacture. For example, a temperature decrease of 2°C may switch synthesis off whereas an increase may promote it. Furthermore, light conditions may also have an effect.

It would be interesting to attempt to define which incubation conditions promote production of the readthrough protein. By comparing extracts of PLRV-infected protoplasts which have been incubated in different temperatures and light intensities, the effects of these on readthrough protein

production and other virus products could be determined.

4.4 THE EFFECT OF A DARK INCUBATION PERIOD ON THE MULTIPLICATION OF PLRV IN PROTOPLASTS

In most cases, protoplasts are cultured in conditions of constant light of varying intensities (Kubo *et al.*, 1975b; Barker and Harrison, 1982; Mayo and Barker, 1983) but it has been reported that they can be incubated in the dark and still support virus multiplication (Reunova *et al.*, 1988; Woolston *et al.*, 1989; Brough *et al.*, 1992). Indeed, virus replication in protoplasts has been shown to occur in light intensities varying from total darkness (Brough *et al.*, 1992) to 4000 lux (Howell and Hull, 1978). Dark incubation conditions tend to be preferred by protoplasts which have been isolated from cells in suspension culture (Sander and Mertes, 1984) but protoplasts isolated from leaf tissue and infected with certain viruses such as geminiviruses are also incubated in the dark (Townsend *et al.*, 1986; Matzeit *et al.*, 1991; Brough *et al.*, 1992). Furthermore, BYDV-PAV has been shown to replicate efficiently in protoplasts isolated from an oat suspension culture and incubated in the dark at 28°C (Dinesh-Kumar *et al.*, 1992). Takebe (1977) reported that the yield of virus in protoplasts decreased when they were cultured in the dark but the addition of sucrose as an energy source increased it. Light intensities above 5000 lux have been reported to be harmful to protoplasts and for tobacco leaf protoplasts infected with TMV, constant light of 1000 lux is said

to result in more virus multiplication than 2000 lux for 16 hrs followed by darkness for 8 hrs (Sander and Mertes, 1984). Additionally, Reunova *et al.* (1988) showed that TMV replication in protoplasts was decreased when the culture was exposed to light conditions other than typical visible light, i.e. darkness, UV light, blue light and yellow light.

To establish the effect of a period of darkness on PLRV multiplication levels in tobacco protoplasts, a series of growth curve experiments was conducted comparing dark conditions with normal light conditions in the incubation of PLRV-inoculated protoplasts.

Initially, a 6 hr dark period was introduced into each 24 hrs of light. ELISA data showed that this appeared to have increased the yield of virus production in protoplasts (Fig. 4.4a). The first protoplast samples taken at 24 hrs post-inoculation showed that more virus was present in the constant light sample. But, in the 48 and 72 hr samples, those which were dark incubated for 6 hrs each day contained a larger quantity of virus. The first sample which had been exposed to a single dark period only may not have had sufficient time for the effect of the dark period on the virus multiplication rate to be noticeable.

In an attempt to further establish the effect this dark period has on virus multiplication, PLRV-inoculated protoplasts were incubated in the light for varying lengths of time and then moved to the dark, and vice versa, incubated in the dark for certain lengths of time and then moved to the light. The samples were incubated for a total of 48 hrs.

The results of this experiment are shown in Fig. 4.4b. Samples which

FIGURE 4.4a: The effect of light conditions on PLRV multiplication in protoplasts.

Protoplasts were inoculated with PLRV virus particles and incubated at 25°C in constant light or in 18 hrs of light alternating with 6 hrs of darkness. Harvesting took place at 24, 48 and 72 hours post-inoculation. Virus concentration in protoplasts was determined by ELISA. The results of three separate experiments were used to determine the standard error of the mean and this is shown as error bars on the graph.

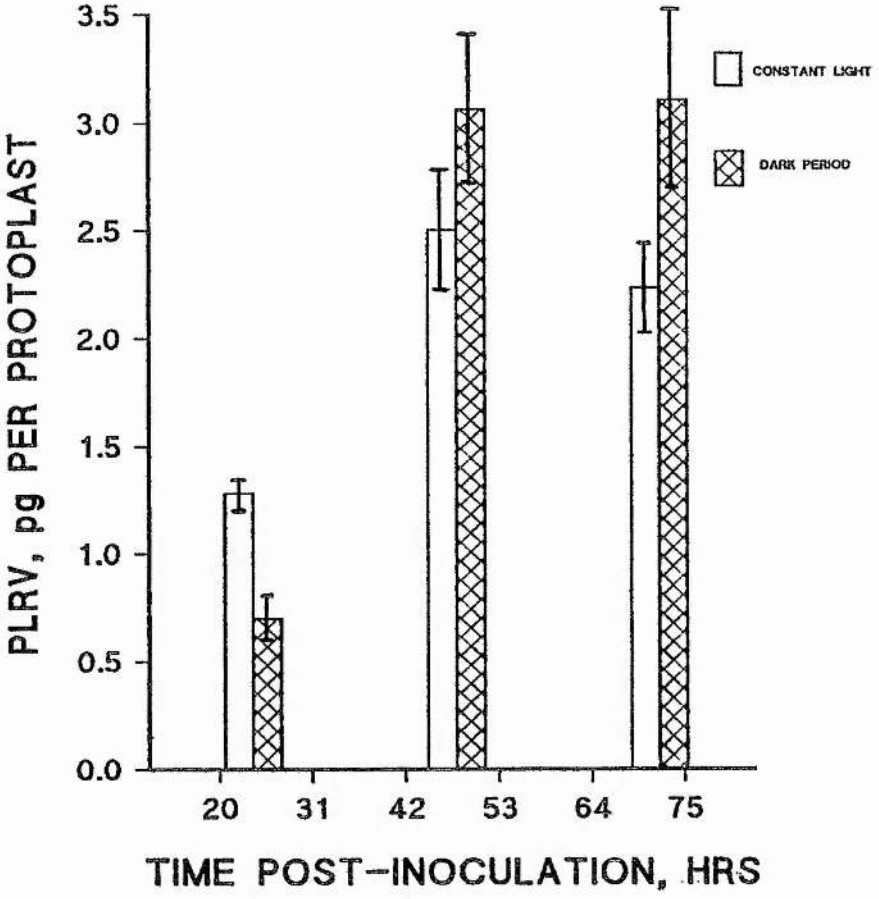
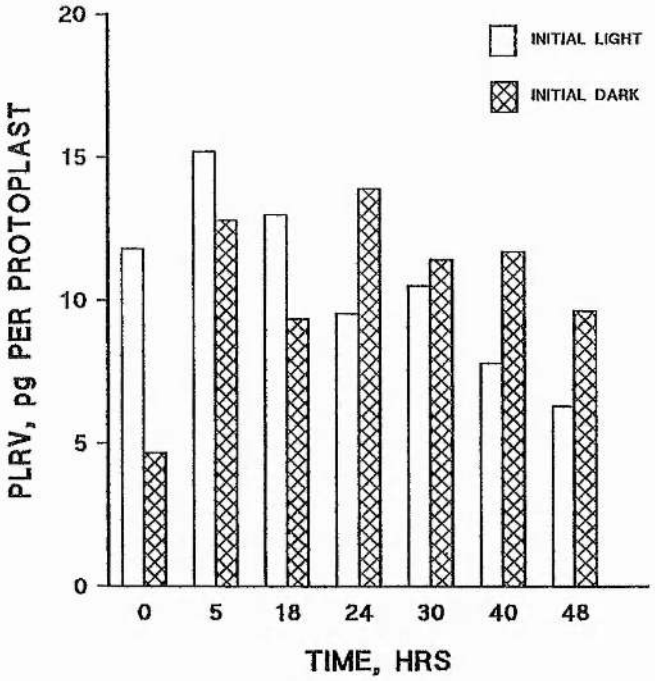

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PLRV, pg PER PROTOPLAST
CONSTANT LIGHT
DARK PERIOD
20
31
42
53
64
75
TIME POST-INOCULATION, HRS

FIGURE 4.4b: The effect of different light conditions on PLRV multiplication in protoplasts.

Protoplasts were inoculated with PLRV virus particles. All samples were harvested at 48 hrs post-inoculation. Half of the samples were initially incubated in darkness and half in light. After periods of 5, 18, 24, 30 and 40 hrs, one sample was moved from light into darkness and vice versa and it remained there until 48 hrs post-inoculation. Two samples were incubated in constant light (0 hrs initial darkness and 48 hrs initial light) and two in constant darkness (0 hrs initial light and 48 hrs initial darkness). Virus content in protoplasts was determined by ELISA. This experiment was repeated at least three times but owing to the variation in protoplast sampling times between the experiments, it has proven impossible to calculate numbers which would accurately reflect the standard error of the mean. Each experiment, however, showed a similar pattern of virus multiplication in protoplasts.


20
15
10
5
0
PLRV, pg PER PROTOPLAST
INITIAL LIGHT
INITIAL DARK
0
5
18
24
30
40
48
TIME, HRS

were incubated continuously in the dark contained substantially higher quantities of virus than those which were incubated continuously in the light. Between these two extremes, those incubated initially in the light for 5-18 hrs appeared to contain more virus than those incubated initially in the dark for this time. However, an initial dark incubation of 24 hrs and longer (up to 48 hrs) yielded higher virus quantities than an initial light incubation of the same time. It appears that virus accumulation, in general, tailed off as the initial time in each light condition increased. This may have been caused by the dramatic change in light conditions having a direct effect on virus multiplication, perhaps causing it to stop for a short time in order for the cells or virus to re-adjust. This may happen in all the samples but those which have been moved from one light condition to another earlier in the incubation probably have enough time to recover and resume virus production at a high level before the end of the incubation period. Those samples which were moved late in the incubation period may not have had enough time, resulting in the manufacture of smaller amounts of virus.

Another experiment involved adding 2% sucrose to the incubation medium of one batch of PLRV-inoculated protoplasts which was dark-incubated and one which was light-incubated. A third batch of PLRV-inoculated protoplasts free of added sucrose was also incubated in continuous light as a control. The protoplasts were incubated for 48 hrs and the results of an ELISA assay showed that the light and dark-incubated protoplasts in the presence of sucrose contained very similar amounts of PLRV, however, the light-incubated batch which did not contain sucrose in its medium

contained a substantially larger amount of virus.

Northern blots of RNA extracted from similar experiments show that the longer the period of light incubation, the more RNA is present (Fig. 4.4c).

Therefore it seems that although more virus accumulates when the protoplasts are incubated in darkness, less RNA is made. It is possible that less unnecessary replication is taking place during dark incubation, ensuring that only the amount of RNA needed for packaging and further replication is made, so that essential nutrients are not consumed to make RNA which will not subsequently be used in the protoplast or packaged, as may happen in protoplasts incubated in constant light conditions.

Light may be having some inhibitory effect on transcription or translation which has allowed the initial synthesis and expression of RNA but as time proceeds, futher synthesis or translation into protein is inhibited.

4.4.1 THE EFFECT OF A DARK INCUBATION PERIOD ON THE MULTIPLICATION OF TRSV IN PROTOPLASTS

To establish if PLRV was unique in its behaviour in these conditions of protoplast culture, a purified preparation of tobacco ringspot virus (TRSV) was used to inoculate protoplasts. Samples were taken at several time intervals from protoplasts which were incubated in light alone and from protoplasts which were incubated in 18 hrs of light and 6 hrs of darkness.

FIGURE 4.4c: Northern blot of RNA extracted from PLRV-infected protoplasts which had been incubated in different light conditions.

RNA was extracted, and Northern blotted, from PLRV- and buffer-inoculated protoplasts which were incubated at 25°C for a total of 48 hrs.

i. PLRV-inoculated samples were incubated in the light for 3, 6, 9, 15, 18, 24, 31 and 36 hrs and then moved into the dark (lanes 1-9 respectively). One PLRV- and one buffer-inoculated sample was incubated in continuous light (lanes 1 and 10 respectively).

ii. PLRV-inoculated protoplasts were incubated in the dark for 3, 6, 9, 16, 20, 31, 41 and 45 hrs and then moved into the light (lanes 2-9 respectively). Lane 10 contains RNA from PLRV-infected protoplasts which were incubated in the light for 48 hrs in medium containing 2% sucrose, lane 11 contains an identical sample which was incubated in the dark for 48 hrs and lane 12 contains PLRV-infected protoplasts which were incubated in light for 48 hrs in medium free of sucrose. Lane 1 contains RNA from buffer-inoculated protoplasts which were incubated in light for 48 hrs. Single arrow heads indicate genomic RNA and double arrow heads indicate subgenomic RNA.

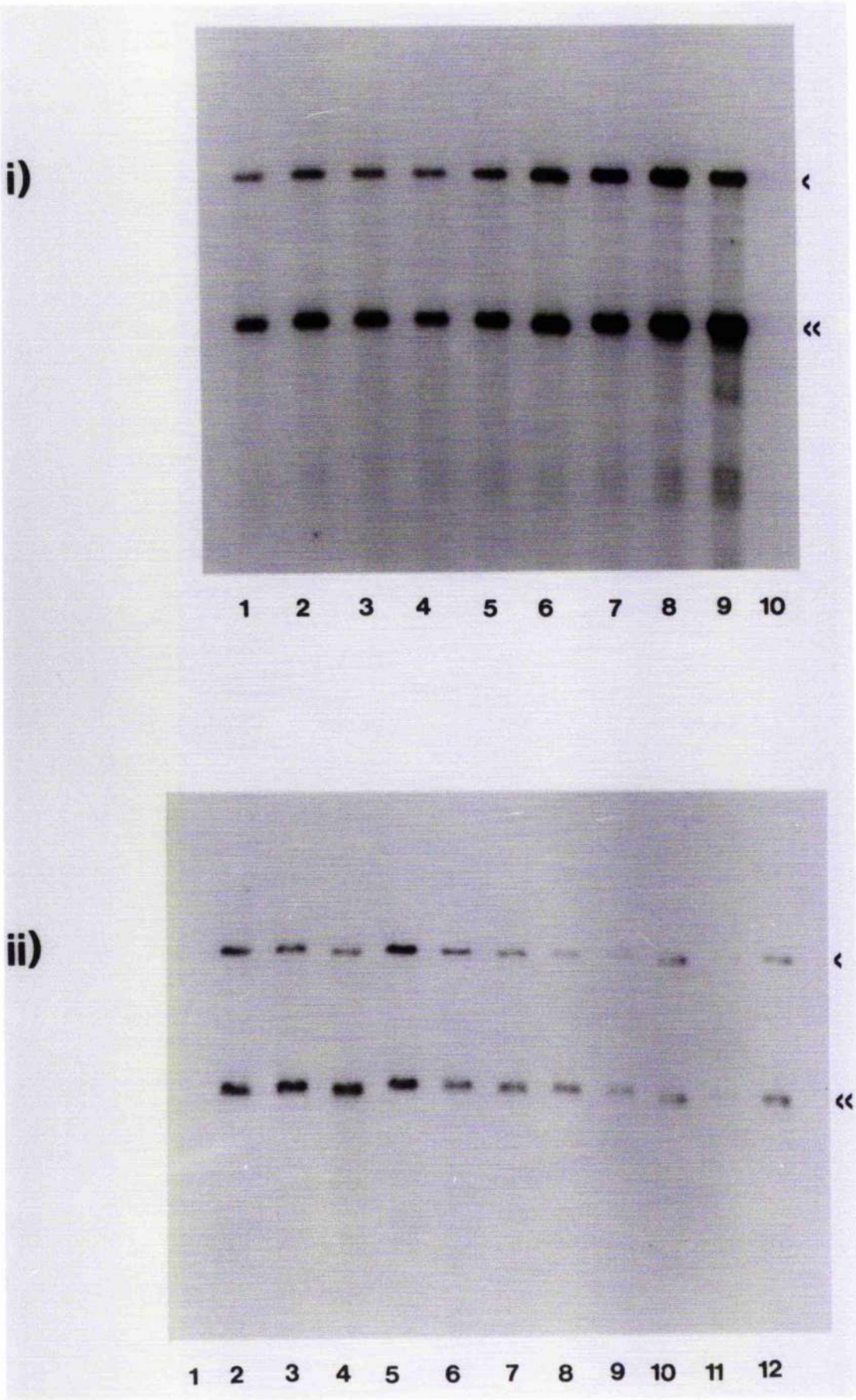
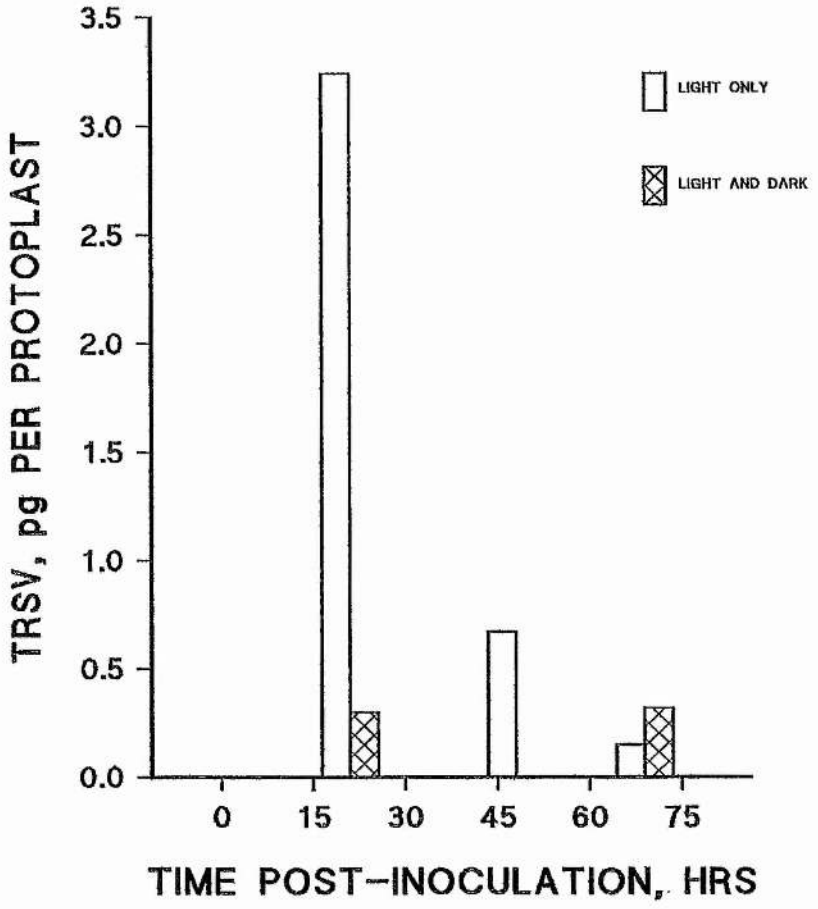

i)
1 2 3 4 5 6 7 8 9 10
ii)
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 4.5: The effect of light on TRSV multiplication in protoplasts.

Protoplasts were inoculated with TRSV virus paticles and incubated in constant light or 18 hrs light alternating with 6 hrs darkness. Virus content in protoplasts was determined by ELISA. This experiment was repeated several times but in most cases, TRSV multiplication was not strong enough to be detected, therefore statistical analysis was impossible.


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TRSV, pg PER PROTOPLAST
LIGHT ONLY
LIGHT AND DARK
0
15
30
45
60
75
TIME POST-INOCULATION, HRS

The results were determined by ELISA using known concentrations of purified TRSV as standards. The results are shown in Fig. 4.5. The figure shows that in the 24 hr sample, there was a significantly larger amount of virus present in the light-only sample compared to the light and dark sample. The amount of virus decreased in both 48 hr samples, but there was again more in the light-only sample. In the final sample taken at 69 hrs post-inoculation, there was still less virus present the light and dark sample now contained slightly more than the light-only sample.

This pattern appears to be completely different to that displayed by PLRV. Reasonably large amounts of virus production seem to have taken place before 24 hrs post-inoculation, but there was a dramatic drop in the amount of virus present which continued until 69 hrs post-inoculation and presumably continued to do so.

TRSV is a nepovirus, and is not limited, as PLRV is, to multiplying only in the phloem tissue. Therefore, incubation containing a dark period may have the opposite effect on this virus, decreasing virus multiplication rather than increasing it owing to the shorter period of exposure to light.

It also appears that TRSV did not multiply very well in general in tobacco protoplasts in this experiment, the protoplasts appearing not to sustain TRSV multiplication after 24 hrs of culture. This suggests that conditions such as higher levels of light, higher or lower temperatures or additional nutrients in the incubation medium may be needed for high levels of TRSV multiplication. Barker and Harrison (1982) described incubation of TRSV infected protoplasts at 20°C whereas the infected protoplasts discussed

here were incubated at 25°C.

PLRV appears to be one of few viruses which can replicate efficiently in dark conditions. There are several explanations for this phenomenon.

i. Large quantities of indigenous metabolites may be present allowing the protoplasts to support virus multiplication for periods of 48 hrs and perhaps longer. Dark incubation followed by light incubation probably forces protoplasts to use these internal substances and subsequently, when fresh metabolites resulting from photosynthesis are also available, there is a new potential for increased virus multiplication. Possibly this is more effective in the protoplasts which were incubated in light and dark cycles.

It was discussed previously that too much light can also inhibit virus multiplication. Different source plant species may each have an optimum light intensity which varies between plants. As stated earlier, protoplasts have been reported to be incubated in light conditions of anything from total darkness (Woolston *et al.*, 1989) to 4000 lux (Howell and Hull, 1978). Light may actually damage protoplasts or the virus, causing loss of replication ability resulting in decreased virus production. The dark period may reflect a chance for the protoplasts to undergo some sort of repair and regain a healthier aspect for when the light period begins.

ii. The conditions of culture of the tobacco plants themselves may have some bearing on the subsequent behaviour of the protoplasts. The plants were incubated in 16 hrs of light and 8 of darkness, so the cells in these plants may be preconditioned to some extent to prefer this type of incubation cycle. so functioning more efficiently in general in light and dark cycles of culture.

iii. The dark period, when included in the culture of the protoplasts may act as some kind of trigger for enhanced virus production. This may be related to it being the phloem tissue which is infected by the virus. Presumably little light reaches this tissue, so the virus is used to multiplying in cells which are not stimulated directly by sunlight. This suggests that it is a property of the virus which results in higher virus production in the dark as compared to the light, and the protoplasts themselves are not directly affected.

Indeed, it appears that it is not only the combination of light and dark conditions which are the cause for the increased levels of virus multiplication. The samples which were incubated solely in the dark, without the provision of another energy source such as sucrose, consistently produced higher levels of PLRV multiplication than those cultured solely in the light. These results may suggest that incubation in light may actually inhibit virus multiplication to some extent but since high levels of PLRV multiplication have been achieved in time course experiments where incubation has been in continuous light, any inhibition would slight. The effect is more likely to be positive rather than negative.

5. PRODUCTS OF PLRV MULTIPLICATION

This chapter deals with PLRV products of multiplication in two main areas:

i. The subcellular location of PLRV multiplication and its products in protoplasts.

ii. The identification of a possible particle intermediate comprising both the subgenomic RNA and the coat protein observed in lysates of PLRV-infected protoplasts.

Results obtained from these experiments may identify a starting point for the determination of which cellular constituents are essential for virus multiplication and how RNA is packaged into virus particles.

5.1 SUBCELLULAR LOCALISATION OF PLRV PRODUCTS IN PROTOPLASTS

The precise location of intracellular PLRV multiplication is unknown. There is evidence that recombination has taken place between PLRV RNA and chloroplast mRNA, suggesting that, at some stage, PLRV can enter the chloroplast (Mayo and Jolly, 1991). Moreover, Schoelz and Zaitlin (1989) have reported that TMV full-length RNA can enter chloroplasts and that once inside, a small proportion is encapsidated by coat protein synthesised internally without the need for subgenomic RNA. Groning *et al.* (1987)

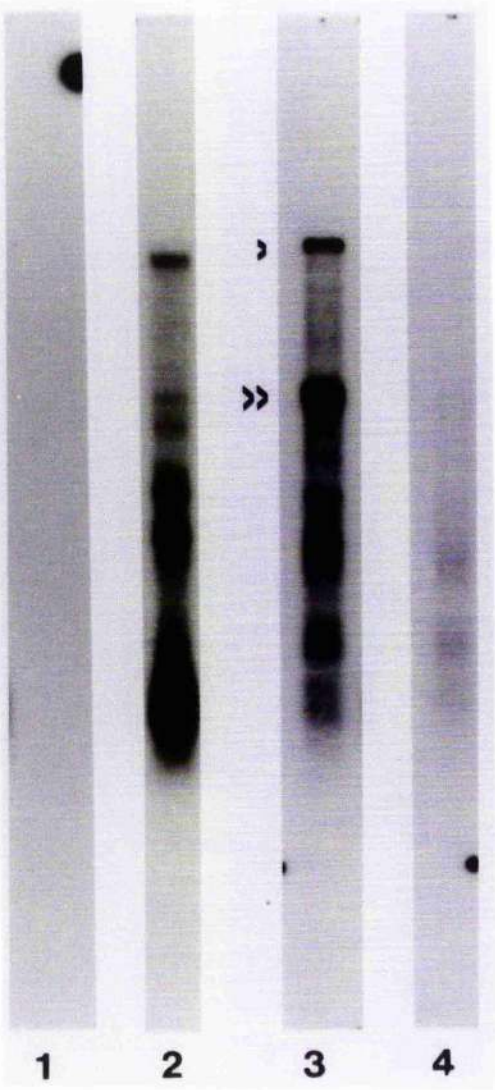
detected abutilon mosaic virus (AbMV) DNA specifically in chloroplasts, in addition to the virus particles being found in the nuclei of the infected cell. It is not clear whether this is an important event or merely an effect of virus multiplication. This experiment attempts to find the location of the majority of PLRV in tobacco protoplasts.

Samples of buffer- and PLRV-inoculated protoplasts were harvested 48 hrs post-inoculation. The protoplasts were pelleted and lysed by vortexing and then centrifuged at 2000 rpm for 7 min to pellet the chloroplasts and other large organelles. The supernate was removed and spun at 10 000 rpm for 10 min to pellet the smaller organelles such as mitochondria. The supernate was again removed and RNA was extracted from each of the three protoplast fractions.

The RNA was blotted and hybridised with probe A (Fig. 2.5). This revealed that both RNA species were present in the pellets obtained from centrifugation at both speeds. In the pellet resulting from centrifugation at 2 000 rpm, more genomic RNA was present than subgenomic (Fig 5.1). In the pellet resulting from centrifugation at 10 000 rpm, less RNA was present in general and, in particular, less genomic RNA was present than subgenomic. There was a large amount of non-specific hybridisation in both tracks. The supernate which remained after the 10 000 rpm spin generally contained too little RNA to be detected by northern blot although in one experiment, a genomic band was observed. Samples of RNA extracted from mock-inoculated protoplasts did not contain any virus-specific bands on northern blots.

FIGURE 5.1: Northern blot of RNA extracted from different protoplast fractions.

RNA was extracted from buffer- and PLRV-inoculated protoplasts after differential centrifugation and northern blotted. Lane 1 contains RNA from buffer-inoculated protoplasts, lane 2 contains RNA from a pellet obtained from high speed centrifugation, lane 3 contains RNA from a pellet obtained from slow speed centrifugation and lane 4 contains RNA from the remaining supernate. A single arrow head indicates genomic RNA and a double arrow head indicates subgenomic RNA.


1
2
3
4

A subsequent experiment was done in which the same procedure was followed with protoplast samples taken at 48 hrs post-inoculation and analysed in an ELISA assay.

This showed that the pellet from the slower spin contained the greatest quantity of virus. This complements the data obtained from the RNA extraction, where this sample appeared to contain more RNA. There are two possible explanantions for this. One is that the RNA and virus particles which sedimented at the slower speed may be attached to or contained within a membrane or organelle which was sedimented at this speed. The second is that the virus may have formed aggregates which sediment at a slow speed. In the RNA extraction from the faster pellet, the RNA which is not attached to large, membranous structures or organelles but may be present within smaller organelles probably pellets at the higher centrifugation speed. RNA which is free in the protoplast is probably degraded rapidly on lysis and free virus particles are too small to be pelleted during either spin.

The explanation for the location of the virus particles may be similar. For some viruses, e.g. geminiviruses and SBMV (Bock, 1974; Goodman and Bird, 1978; Osaki and Inouye, 1981; Bock and Harrison, 1985; Buck and Coutts, 1985; Tremaine and Hamilton, 1983) particles are known to aggregate in the nucleus of the cell, and this organelle is large enough to sediment at the slower speed. Particles which are not in an aggregate nor are located in a cellular organelle are unlikely to sediment at either speed. Therefore, when RNA was extracted from the remaining supernatant fluid, only genomic RNA would be found since probably only virions were present.

These two pieces of evidence suggest that most virus particles and unencapsidated RNA are membrane-associated or contained within the larger organelles. Gill and Chong (1981) reported that for BYDV, RNA replication may take place in double-membraned vesicular cytoplasmic stuctures and the RNA sedimentation patterns described here appears to agree with this. The experiment discussed here was designed to attempt not to lyse internal membranes before the extraction steps so that virus-specific products sedimented with the structure they were associated with. Therefore, if membranous vesicles were present, they may have been sedimented intact, explaining why most of the virus RNA was obtained from the slow speed spin.

The PLRV/chloroplast RNA recombination event was reported to take place at the 5'-end of the genomic RNA, but it was not known at which point in the PLRV multiplication cycle the recombination had taken place and what role the chloroplast played (Mayo and Jolly, 1991). It was also unknown in what form the viral RNA had entered the chloroplast, as a virion or as free RNA. As mentioned previously for TMV, only genomic RNA could enter tobacco cloroplasts. Virions were shown not to enter the chloroplast and it was proposed that the subgenomic RNA did not contain the required sequences for such transport (Schoelz and Zaitlin, 1989).

To establish the intracellular location of PLRV multiplication more precisely, each organelle and membranous structure should be isolated individually and the RNA blotted and probed. However, the protoplast model system may not be ideal for this purpose since it is thought that the isolation

procedure may interfere in some way with the intracellular organisation of the protoplasts (Harrison and Mayo, 1983). Moreover, it may be discovered that neither the virus nor the RNA is found inside any organelle.

5.2 THE SEDIMENTATION BEHAVIOUR OF PLRV PROTEIN IN PROTOPLASTS

When the purified particles of luteoviruses are centrifuged through a sucrose gradient, a single sedimentable component is generally found which has a sedimentation coefficient of between 104*S* - 127*S* (Waterhouse *et al.*, 1988). PLRV particles have been reported to have a sedimentation coefficient of 115*S* (Harrison, 1984). However, Hewings and D'Arcy (1986) found, in addition to the main component, a lighter sedimentable component in a Californian isolate of BWYV. This component was reported to have a sedimentation coefficient of 62*S* and contain empty virus particles which were found to be non-infectious. Similarly, Proll *et al.* (1985) found a second sedimentable component associated with an isolate of BYDV. This also appeared to contain non-infectious virus-like particles with a sedimentable coefficient of 53*S*.

5.2.1 GRADIENT CENTRIFUGATION OF LYSATES OF INFECTED PROTOPLASTS

PLRV- and mock-infected protoplasts were harvested and lysed by crushing with a micro-pestle or extensive vortexing. The protoplasts were centrifuged for 10 min at 10 000 rpm, the supernate removed and loaded on to a 10%-40% sucrose gradient. Virus particles (0.5 µg) were added to the healthy protoplast lysate and loaded onto a second sucrose gradient. The gradients were spun at 45 000 rpm for 50 min and separated into 7 drop fractions by upward displacement through an ISCO fractionater. The antigen content of each was determined by ELISA.

Near the bottom of the gradient which contained the purified virus particles, there was a precise and well-defined peak (Fig. 5.2a). This corresponded to the sedimentable component of luteovirus particles as described by Waterhouse *et al.* (1988). Antigen in the lysate of infected protoplasts, however, sedimented as 2 main components, one at the same position as that found for virus particles and a second, present as a peak near the top of the gradient, presumably corresponding to a structure lighter than virus particles (Fig. 5.2a).

The fractions which contained the material causing these strong reactions in the ELISA assay were examined using electron microscopy. Carbon-coated grids were coated with antibody directed against PLRV and incubated with the sample fraction for periods of 1 hr or more at 37°C. Subsequent observation of these grids using the electron microscope showed

FIGURE 5.2a: ELISA profile of protoplast lysates after sedimentation in a sucrose gradient.

Protoplasts were inoculated with PLRV virus particles and incubated for approximately 40 hrs. Samples were lysed and the lysate loaded onto sucrose gradients. Gradients were centrifuged, fractionated by upward displacement and the antigen content in each sample assayed by ELISA. Buffer-inoculated protoplasts were lysed and 0.5µg of PLRV virus particles was added to the lysate, the above procedure was then followed.

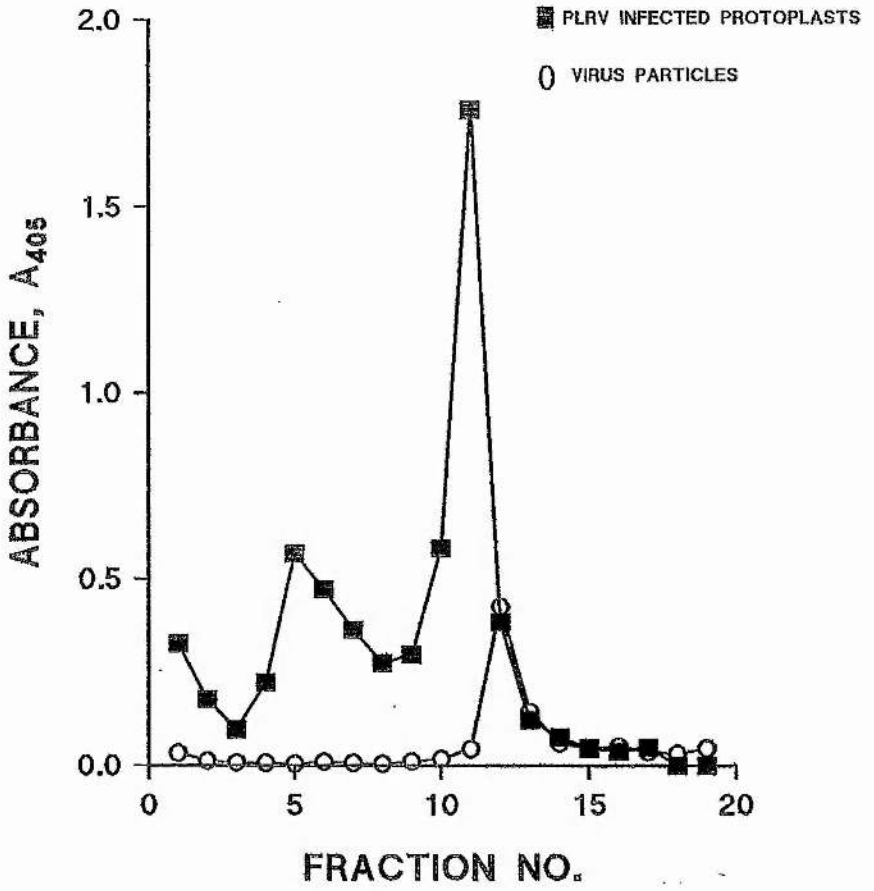
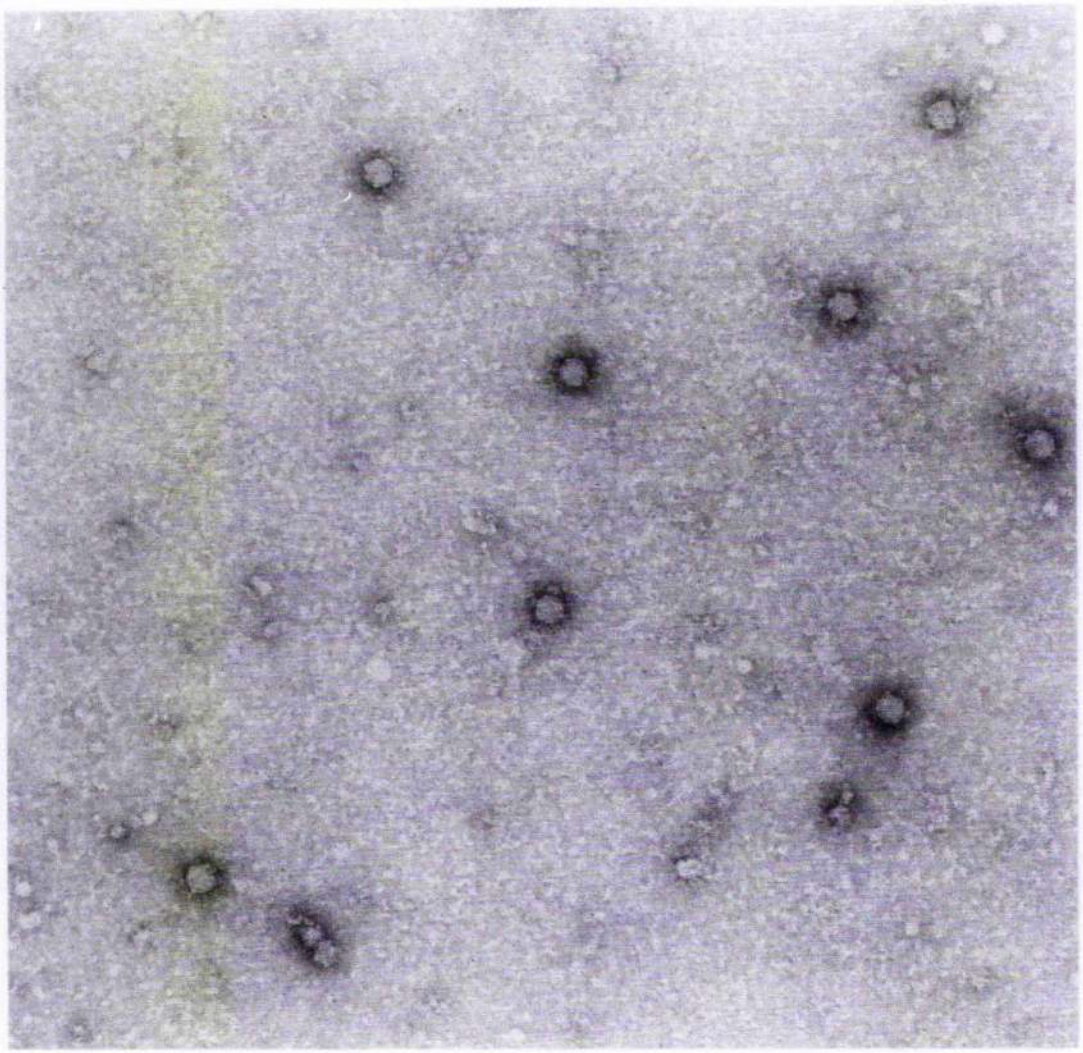

PLRV INFECTED PROTOPLASTS
VIRUS PARTICLES
ABSORBANCE, A_{405}
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
FRACTION NO.

FIGURE 5.2b: Electron micrograph of PLRV virus particles retrieved from a sucrose gradient

A quantity of 0.5 μg of PLRV virus particles was added to a lysate of healthy protoplasts and the mixture was loaded onto a sucrose gradient. The gradient was centrifuged and fractionated as described and the antigen-containing fractions located by ELISA. Drops of the particular fractions were placed on carbon-coated grids, stained with uranyl acetate and used for electron microscopy. The magnification on the micrograph was 39 000.



that in lysates of healthy protoplasts which contained purified virus particles, these appeared to be present in quite large numbers, approximately 5-10 per field of view (Fig. 5.2b). The bottom component of the gradient containing the lysate of the PLRV-infected protoplasts contained virus particles of which many appeared to be irregular in shape having lost the well-defined isometric morphology typical of luteoviruses. The particles were not very abundant. The lighter component which reacted in the ELISA did not appear to contain anything recognisable as being virus-related.

The samples which were loaded onto the sucrose gradients were also assayed for virus content, by EM and ELISA, and were found to contain large quantities of virus particles which were intact and, morphologically speaking, in good condition. The amount of virus in these protoplast lysates was estimated to be between 0.5 - 1.3 μg. An almost equivalent, and sometimes larger, amount of virus was found in the material pelleted when the lysed protoplasts were centrifuged.

5.2.2 THE EFFECTS OF DIFFERENT CONDITIONS OF PROTOPLAST LYSIS ON PLRV GRADIENT PROFILES

Because of the inability to find many intact virus particles in the bottom component in the sucrose gradient, and to find any virus particle-like structures in the top component using EM, experiments were performed to test the effect of the conditions in which the protoplasts were lysed. This was

done using several different buffers and lysing techniques to attempt to determine if the particle morphology or integrity was affected in any way.

Initially, the protoplasts were lysed in the same PBS/Tween solution which was used as extraction buffer for the subsequent ELISA assays. Although this gave the same ELISA profile (as in Fig. 5.2a), few particles were observed using the electron microscpe. Nothing was visible in fractions containing the lighter component. Tween, being a detergent, was used to lyse the protoplast membrane and give easier and more efficient lysis but it may also have promoted instability of the virus particles, preventing many from surviving the gradient intact. Therefore, Tween was removed from the buffer and PBS, pH 7.0, was used instead. This appeared to make no noticeable difference to the quantity or quality of the resulting virus particles or ELISA profile compared to the solution containing Tween.

When 0.01M-phosphate buffer, pH 7.0 alone was used no significant difference was observed in ELISA profile, or virus particle quality. This buffer was used for all the remaining gradient experiments.

5.2.3 THE EFFECT OF SUCROSE ON PARTICLE STABILITY

The possibilty of sucrose affecting the stability of the virus particles and playing a role in preventing their adherence to antibody-coated grids was considered. The relevant fractions containing virus particles were microdialysed. A 20µl quantity of each fraction was placed on a Millipore

filter, pore size 0.025 μm, floated on 0.01 M-phosphate buffer and left at room temperature for 1-2 hrs. This procedure was intended to remove the sucrose from the fraction sample; however, the virus particles appeared to be no different compared to those in the presence of sucrose, as seen by electron microscopy.

To attempt to test further the effects of sucrose on virus particles, some particles were added to a sucrose solution similar to the gradient solutions used and incubated for several hours at room temperature. The particles were checked for denaturing effects of the sucrose. Those which had been incubated in the sucrose solution were slightly irregular in shape and fewer were completely intact compared to the control particles incubated for the same length of time in phosphate buffer (Figs. 5.3a,b). Nevertheless, the difference between the two samples was not severe enough to explain the very low quantities of virus detected in the gradient samples.

5.2.4 THE EFFECT OF DIFFERENT BUFFERS ON THE CONDITION OF VIRUS PARTICLES

Massalski and Harrison (1987) noted that PLRV virus particles were degraded when incubated in 0.05 M-sodium carbonate buffer, pH 9.6, (coating buffer) so that when a sample was examined using the electron microscope, after 3 hrs of incubation at 37°C, no particles were found. Moreover, when purified particles which had been incubated in coating

FIGURE 5.3a: Electron micrograph of PLRV virus particles after incubation in phosphate buffer.

PLRV virus particles were incubated in phosphate buffer for 3 hrs at room temperature. Samples were then placed on carbon coated grids, stained with uranyl acetate and examined. The magnification on the micrograph was 39 000.

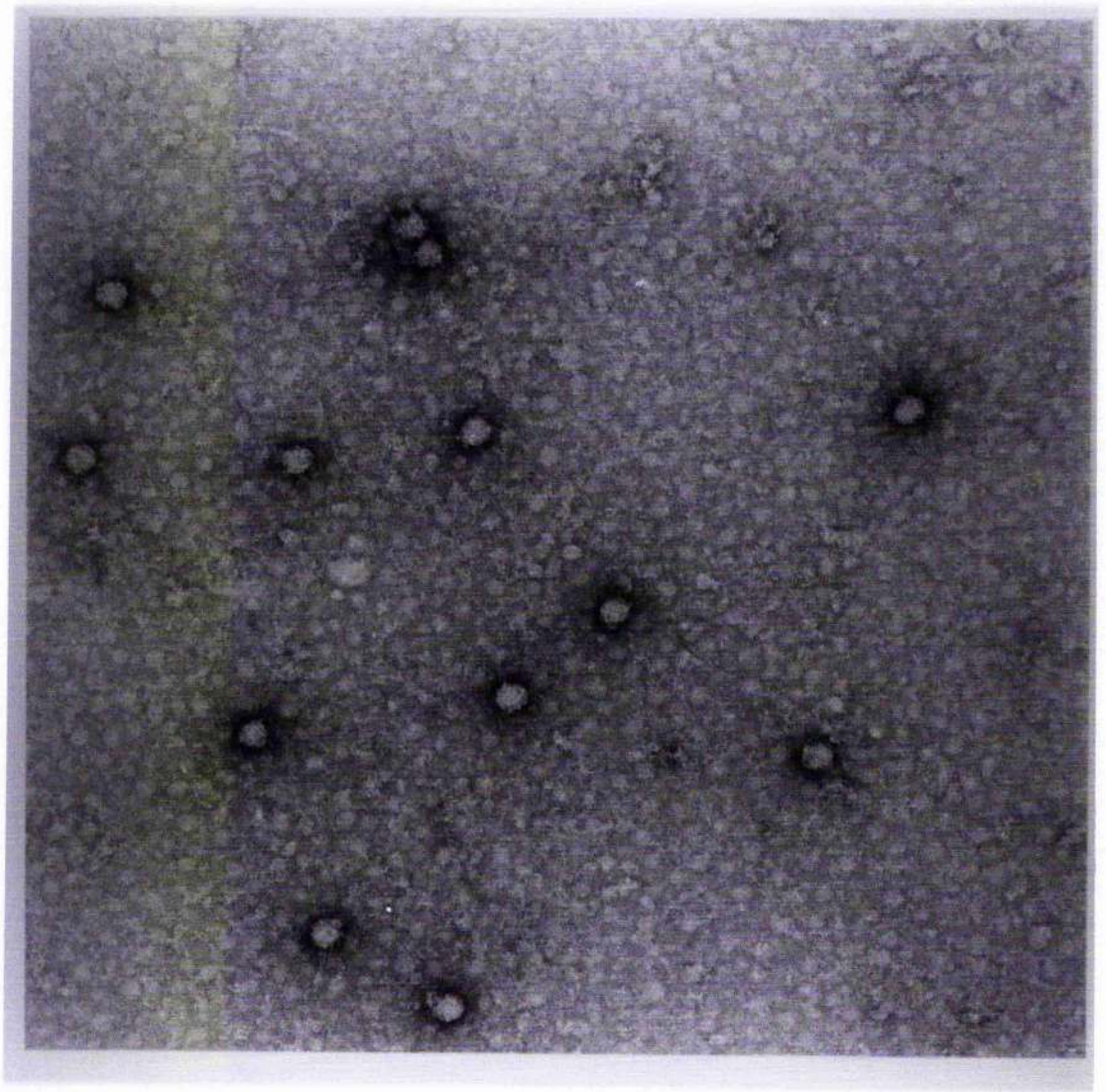
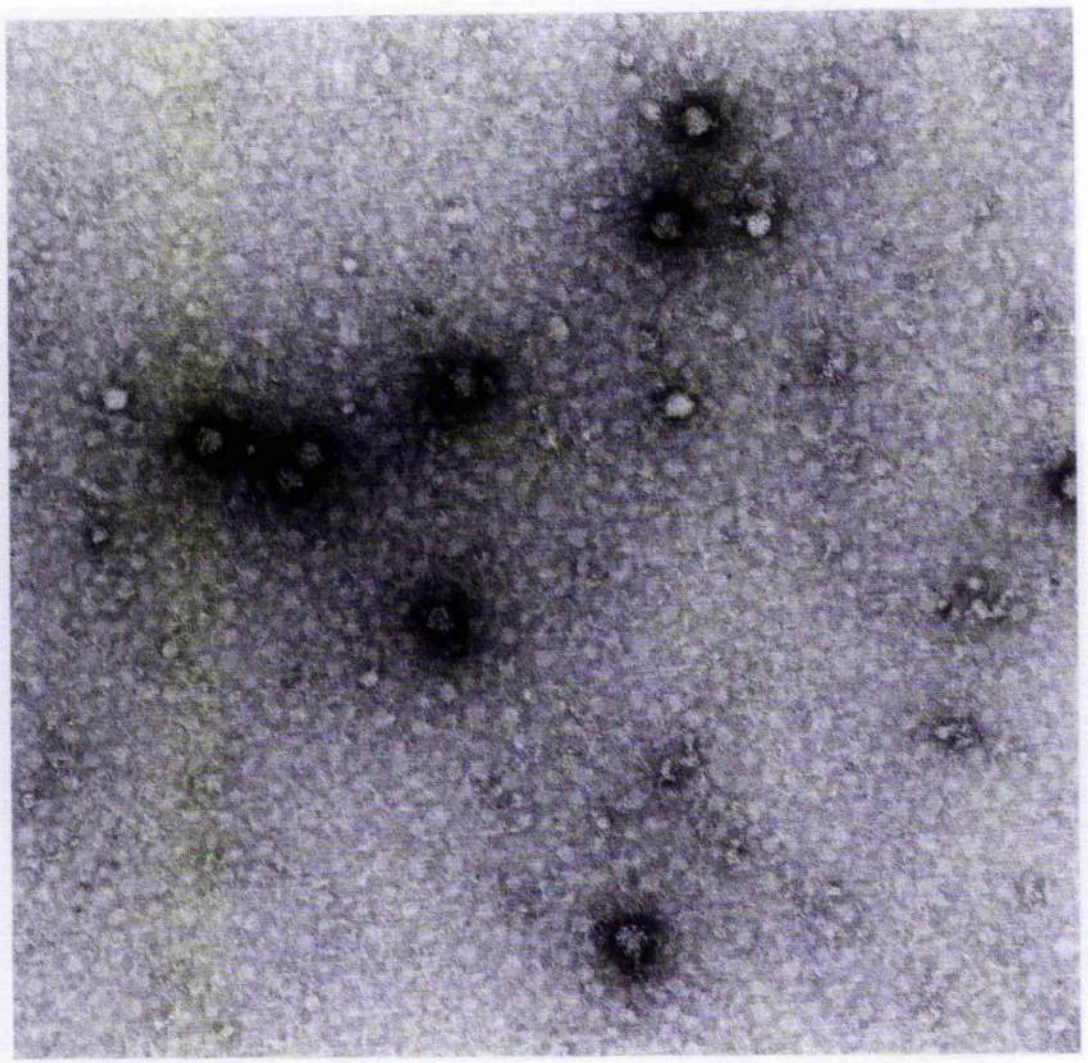


FIGURE 5.3b: Electron micrograph of PLRV virus particles after incubation in phosphate buffer containing sucrose.

PLRV virus particles were incubated in phosphate buffer for 1 hr at room temperature in containing sucrose. Samples were then placed onto carbon coated grids, stained with uranyl acetate and examined. The magnification on the micrograph was 39 000.



buffer were compared to some which were incubated in PBS and sedimented in sucrose density gradients, those which had been incubated in PBS produced the typical sharply defined single component in the adsorption profile but those incubated in coating buffer contained an ill-defined and much smaller main component and an increase in the amount of material at the top of the gradient.

An attempt was made to clarify which buffer conditions were best for the stability of the particles. Virus particles were extracted from leaf tissue by grinding in the presence of carborundum powder in one of several different buffers. The extract was left in buffer at room temperature for periods of between 1 and 24 hrs . The buffers used were;

i. Phosphate buffer, 0.01 M, pH 7.0.

ii. PBS, 0.1 M, pH 7.0.

iii. Tris-HCl, 50 mM, pH 7.0.

iv. Tris-HCl, 50 mM, pH 7.0, containing 5 mM-$CaCl_2$, 5 mM-$MgCl_2$.

v. Tris-HCl, 50 mM, pH 7.0, containing 10 mM-EDTA.

Over a 24 hr period the best results were obtained in the sample extracted with PBS. It appeared to contain the most particles at the end of the incubation period. The Tris-HCl buffer when used alone contained a large amount of particles initially but this number decreased substantially with time. Samples in buffer (i) also showed reasonably good results. They contained slightly fewer particles than samples extracted in PBS but the particles present were retained intact and with good isometric morphology for as long as the test continued.

Therefore, it appears that the buffers used were not the reason for the low numbers of particles which were present in the gradient fractions.

5.2.5 THE EFFECT OF PARAFORMALDEHYDE TREATMENT ON PARTICLE STABILITY

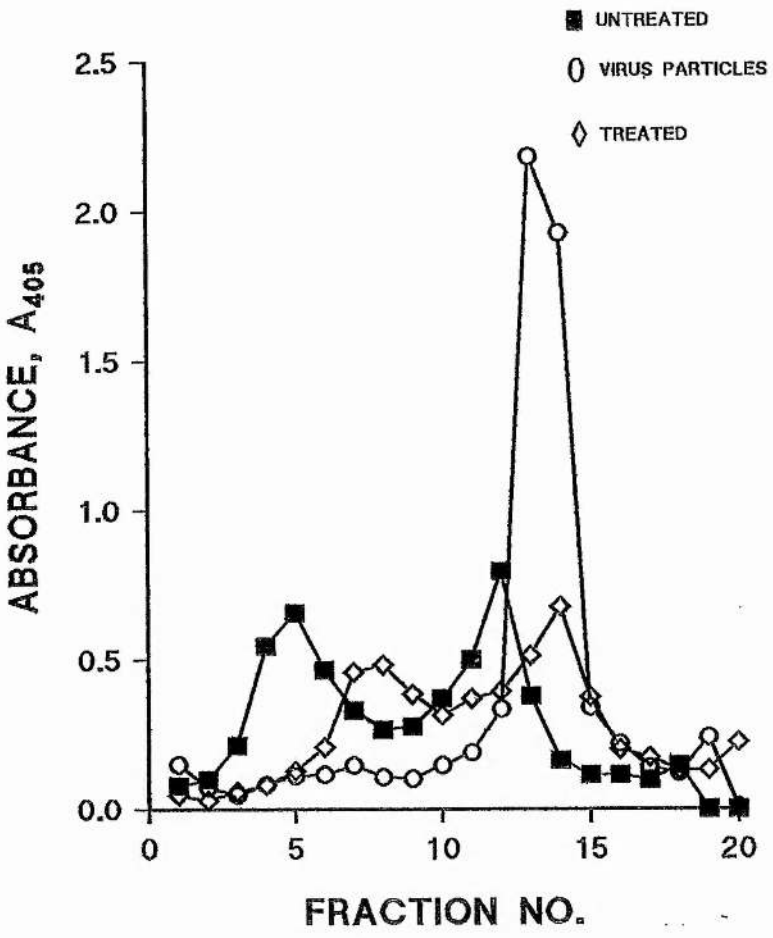
A further experiment was performed to attempt to prevent the virus particles from being degraded during centrifugation. This involved "fixing" the virus particles present in the protoplast lysate. Paraformaldehyde was added to the sample to give a final concentration of 0.1%. The sample was incubated at room temperature for 10 min and glycine was then added to a final concentration of 0.1%. This procedure was intended to lend greater stability to the virus particles and render them non-degradable.

The treated and untreated loading samples were checked by EM for virus particle content before being loaded onto gradients. The control sample contained the usual quantity of virus particles but the sample which had been treated with paraformaldehyde appeared to contain nothing visible using EM. The gradients were run nevertheless and the subsequent ELISA assay showed that both samples had the typical profile (Fig. 5.4). The sedimentable components in the treated sample, however, sedimented more quickly than in the untreated sample, appearing 1-2 fractons lower in the gradient.

This difference between the antigenic profiles of the treated and untreated samples may indicate that the virus particles in the treated sample

FIGURE 5.4: The effect of paraformaldehyde treatment on the ELISA profile of a sucrose gradient containing a lysate of PLRV-infected protoplasts.

PLRV- and buffer-inoculated protoplast samples were lysed as described previously. One sample of each was treated normally and one PLRV-inoculated protoplast lysate was treated with paraformaldehyde. The samples were loaded onto sucrose gradients, centrifuged, fractionated and the antigen content determined by ELISA.


UNTREATED
VIRUS PARTICLES
TREATED
2.5
2.0
1.5
1.0
0.5
0.0
ABSORBANCE, A_{405}
0
5
10
15
20
FRACTION NO.

have been protected against damage during centrifugation. The treatment may have prevented swelling of the particles which would have resulted in slower sedimentation. Particle swelling has been observed when samples of BMV virus particles were treated with 10 mM-EDTA and 0.2 M-KCl at pH7.5 (Johnson and Argos, 1985) and the resulting sedimentation coefficient which was obtained for these particles was much lower than the normal value, 78*S* compared to 88*S*. This may suggest that retrieving the virus particles from the infected protoplasts and centrifuging them through a sucrose gradient causes swelling, or that swelling has taken place before loading. A change like this in the structure of the virion also implies a loss of particle stability, so could account to some extent for the low numbers of particles found using EM.

It is also possible that many of the particles are in an unstable condition after assembly in protoplasts. The time span of infection and multiplication are much shorter here than in intact plants and may be too short for the synthesis of completely stable virions. Sucrose gradients are often used in the purification procedures of many plant viruses. Damaged or structurally different particles are sedimented to a different position on the gradient from the normal, intact particles, but very much larger quantities of virus particles are being dealt with compared to the relatively tiny quantity present in protoplast lysates. The initial proportion of damaged to undamaged particles may be similar but when only a small quantity is involved, more damage may take place during sedimentation.

Moreover, the lighter component, for which no virus-like structure

was observed using the electron microscope, may be a highly unstable transient structure which would normally be degraded in the cell during the normal multiplication procedure. It may, therefore, be a particle intermediate.

Furthermore, when PLRV virus particles were extracted out of infected leaf tissue and subjected to sucrose gradient centrifugation, there did not appear to be a lighter, top component present (Fig. 5.5). This supports the idea that this component which reacts strongly in the ELISA assay, when extracted from infected protoplasts is very unstable. When a longer infection process is underway, it may be present in such small quantities, for such a short period of time that it is not detectable.

5.2.6 THE DISTRIBUTION OF RNA IN FRACTIONS OF PLRV-INOCULATED PROTOPLAST LYSATES WHICH HAVE BEEN SUBJECTED TO GRADIENT CENTRIFUGATION

After the lysates had been centrifuged and fractionated, a dot blot was made with 3µl of each fraction. After the dots had been made, the nitrocellulose blot was allowed to air dry for about 1 hr and hybridised as for a northern blot. The probes described in Fig. 2.5 were used to detect PLRV-specific RNA.

The RNA followed a similar distribution pattern to the antigen content in the gradient (Fig. 5.6). The healthy protoplast lysate to which PLRV virus particles were added contained clear dots in the fractions which reacted in the

FIGURE 5.5: The ELISA profile of PLRV-infected potato tissue after sedimentation through a sucrose gradient.

A sample of PLRV-infected leaf tissue was taken from a potato plant. It was ground in phosphate buffer, centrifuged to remove particulate material and the supernatant was sedimented through a sucrose gradient. The gradient was fractionated and the PLRV content in each fraction assayed by ELISA.

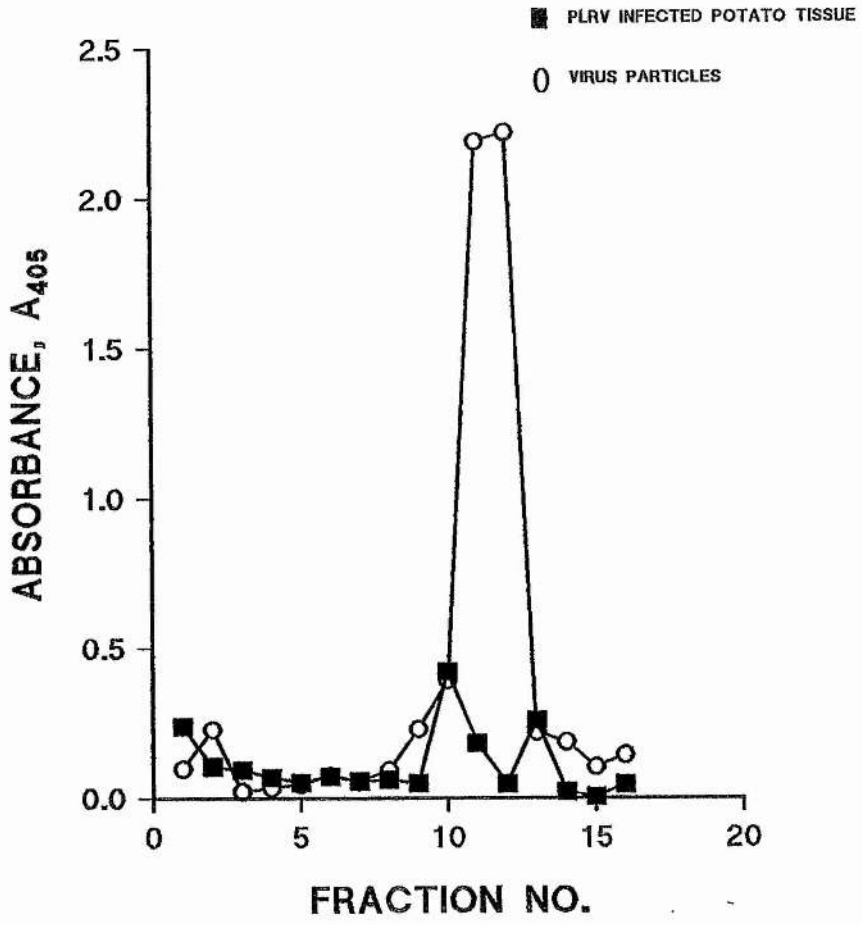
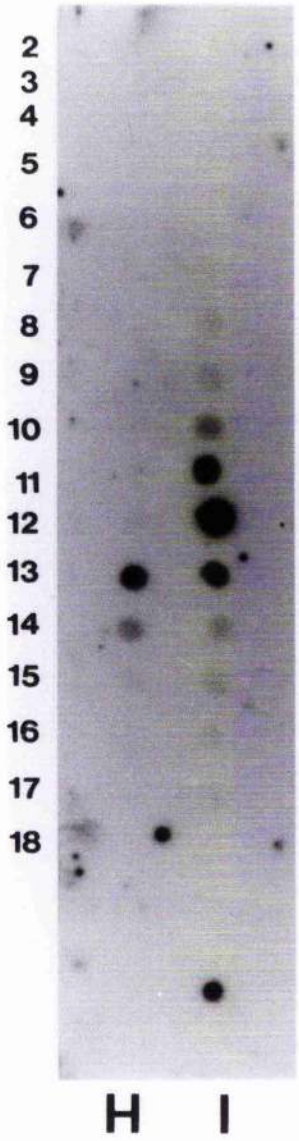

PLRV INFECTED POTATO TISSUE
VIRUS PARTICLES
ABSORBANCE, A_{405}
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
FRACTION NO.

FIGURE 5.6: Dot blot of RNA distribution in a sucrose gradient containing lysates of buffer- and PLRV-inoculated protoplast lysates.

Buffer- and PLRV-inoculated protoplasts were lysed and the lysates were sedimented through sucrose gradients. The gradients were fractionated and 3µl of each fraction was spotted onto a piece of wet nitrocellulose paper. This was then treated as a northern blot. Lane H contains the buffer-inoculated protoplast lysate containing 0.5 µg PLRV virus particles and lane I contains the PLRV-inoculated lysate. The numbers denote fractions, number 2 being the top of the gradient.


2
3
4
5
6
7
8
9
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11
12
13
14
15
16
17
18
H
I

ELISA. In the Figure shown, fraction numbers 11-14 contained the PLRV-specific RNA and particles. The PLRV-infected protoplast lysate contained dots starting from fraction number 6, approximately where the top component was found on the ELISA profiles, and the dots continued until fraction number 17. The darkest of these dots, indicating the presence of the most RNA, was observed at fraction number 11. The virus particle peak was found in this fraction on the ELISA profile.

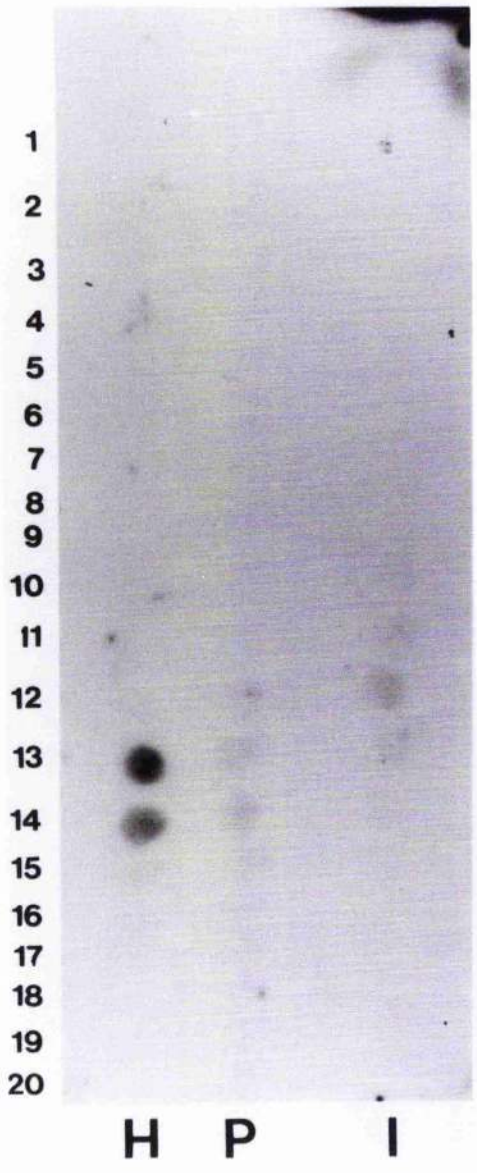
5.2.7 THE EFFECT OF PARAFORMALDEHYDE TREATMENT ON RNA DISTRIBUTION IN FRACTIONS OF PLRV-INFECTED PROTOPLAST LYSATES

When a dot blot was made for the sample which was treated with paraformaldehyde as described in section 5.2.5, dots were present in fractions 11-16 of the lysate of PLRV-infected protoplasts (Fig. 5.7, lane P). In the gradient which had been loaded with purified virus particles, the dots were in fractions 12-15 (Fig. 5.7, lane H). However, in the control sample which was a PLRV-infected protoplast lysate which had not been treated with paraformaldehyde, dots were present in fractions 9-14, the darkest being at fraction number 12 (Fig. 5.7, lane I). RNA was not detected higher in the gradient.

This result corroborates that obtained with the ELISA assay, that the particles in the treated sample are sedimenting at the same rate as the purified

FIGURE 5.7: Dot blot of RNA distribution in a sucrose gradient containing buffer- and PLRV-inoculated protoplast lysates and a lysate of PLRV-inoculated protoplasts which had been treated with paraformaldehyde.

Buffer- and PLRV-inoculated protoplasts were lysed. PLRV-inoculated protoplast lysates were i) treated with paraformaldehyde and ii) untreated (control sample). Each lysate was sedimented through a sucrose gradient. The gradients were fractionated and 3µl of each spotted onto a piece of wet nitrocellulose paper. This was then treated as a northern blot. Lane H contains the buffer-inoculated protoplast lysate containing 0.5 µg PLRV virus particles, lane P contains the treated PLRV-inoculated lysate and lane I contains the untreated PLRV-inoculated lysate. Numbers denote fractions, fraction 1 being the top of the sucrose gradient.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
H
P
I

virus particles and the untreated particles are sedimenting more slowly, indicating that the treatment interferes with the sedimentation behaviour of the virus particles.

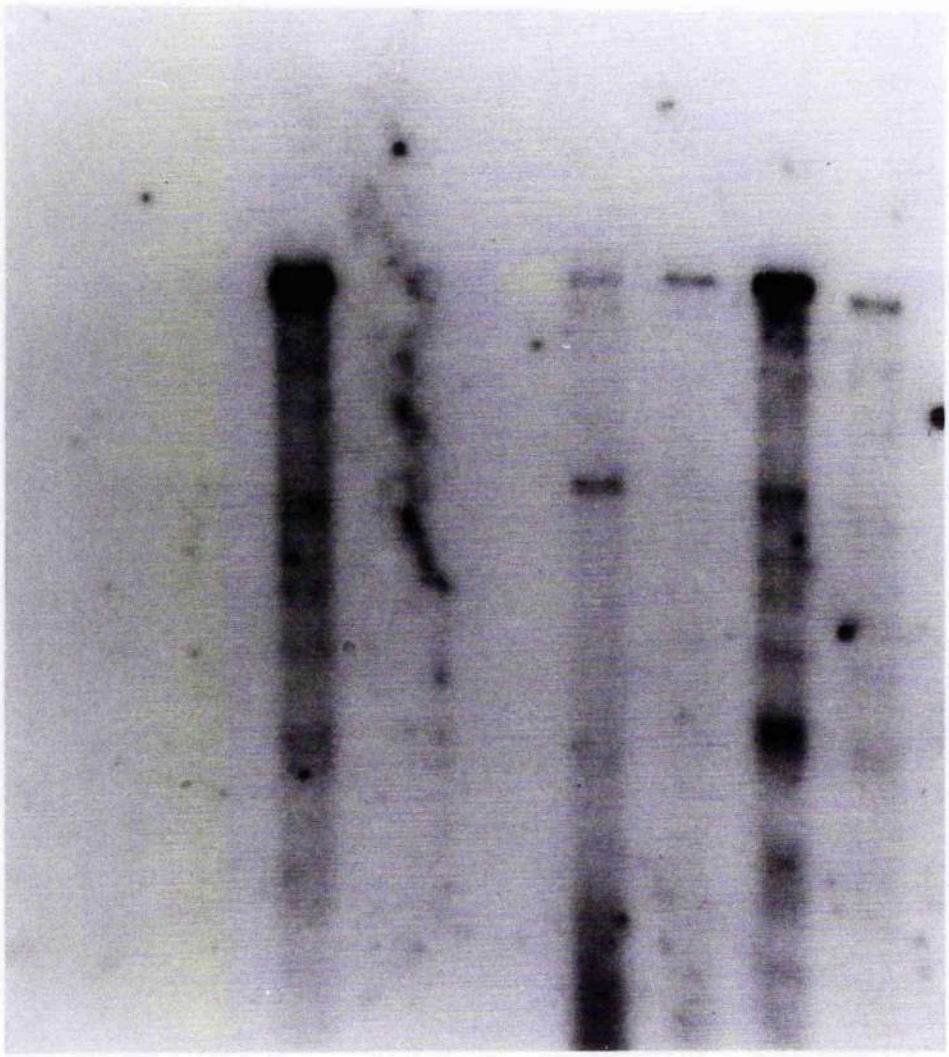
So it appears that the RNA is distributed with the protein which is detected in the ELISA assay, the most RNA being found in the fraction which also contains the most protein.

5.2.8 THE DISTRIBUTION OF GENOMIC AND SUBGENOMIC RNA IN LYSATES OF PROTOPLASTS WHICH HAVE BEEN SUBJECTED TO SUCROSE DENSITY CENTRIFUGATION

Although the dot blot showed where virus RNA was in the gradient and that it appeared to be strongly associated with the virus protein sedimentation behaviour, it could not show which species of RNA were present; genomic, subgenomic or both, or if it was intact or fragmented. To address this question, fractions which had been recovered from sucrose gradients loaded with healthy lysate plus purified particles and PLRV-infected protoplast lysate were bulked into 4 groups per gradient. The fraction groups were 1-5, 6-10, 11-15 and 16-20. RNA was then extracted from these groups. When the samples were blotted (Fig. 5.8), it appeared that there was mainly subgenomic RNA present in fractions 1-5 (Fig. 5.8, lane 5), there was a small amount of genomic RNA present in fractions 6-10 (Fig 5.8, lane 6), there was a large amount of genomic present in fractions

FIGURE 5.8: The distribution of RNA species in the fractions of sucrose gradients containing protoplast lysates.

Buffer- and PLRV-inoculated protoplasts were lysed and the lysates were sedimented through sucrose gradients. A quantity of 0.5 μg of PLRV virus particles was added to the buffer-inoculated protoplast lysate before centrifugation. After centrifugation RNA was extracted from bulked groups of fractions and northern-blotted. Lanes 1-4 contain the buffer-inoculated protoplast lysate fractions containing the PLRV virus particles and lanes 5-8 contain the PLRV-inoculated protoplast lysate fractions. For each lysate the fractions were bulked into groups of fraction numbers 1-5, 6-10, 11-15 and 16-20, number 1 being the top of the gradient. A single arrow head indicates genomic RNA and a double arrow head indicates subgenomic RNA.


1
2
3
4
5
6
7
8

11-15 (Fig. 5.8, lane 7), with a small amount of subgenomic, and there was a small amount of genomic present in fractions 16-20 (Fig. 5.8, lane 8).

Because subgenomic RNA appears in the first 5 fractions at the top of the gradient, some kind of protective protein structure is probably associated with it. It is highly unlikely that a piece of RNA such as this could have survived centrifugation intact through a non-sterile sucrose gradient, unless protected in some way. Since these gradient fractions contain the top component of the virus which reacts in ELISA assays, this protection could be in the form of the coat protein. Because the subgenomic RNA is used as a template to express the viral coat protein, it is possible that the RNA and protein become associated in such a way that the protein, whether completely or partially synthesised, protects the RNA.

Moreover, it appears from the RNA in fractions 11-15 that a small quantity of the subgenomic RNA is being encapsidated with the genomic RNA.

In control gradients loaded with the healthy lysate plus purified particles, RNA was present in only one group of fractions, 11-15 (Fig. 5.8), and only the genomic RNA was present.

The RNA present in each fraction group was in good condition and there had been no apparent degradation.

A possible explanantion for the presence of virus particles which contain subgenomic RNA in addition to genomic RNA in protoplast preparations and apparently not in purified virus preparations is that when virus particles are purified from plant tissue, very stringent conditions are

used to ensure the most efficient yield possible. If abnormal virus particles are present and are even slightly unstable, it is probable that they will not survive the purification procedure. Hence it is unlikely that virus particles containing both RNA species would be present in the purified virus preparation.

5.2.9 CALIBRATION OF SUCROSE GRADIENTS WITH ArMV AND TRSV

The sedimentation coefficient of PLRV virus particles is 115*S*. However, the sedimentation coefficient of the lighter component which has been found in lysates of infected protoplasts is unknown. To try and establish a reasonable estimate for the sedimentation coefficient of this component and characterise further its contents, purified preparations of TRSV virus particles and ArMV virus-like particles were subjected to sucrose gradient centrifugation under the same conditions as described previously for PLRV.

Both of these viruses are nepoviruses and have isometric particles of similar size and shape to PLRV (Martelli, 1991). Therefore, they are likely to sediment similarly to PLRV and could be used in gradient calibration.

Quantities of 10-20 μg of particles of each virus were loaded onto the gradients in 0.01 M-phosphate buffer, pH 7.0. After sucrose gradient centrifugation viral components were visualised by exposure of the gradient to a vertical beam of light. The virus particles appeared as a light scattering

band.

TRSV appeared only to have one light scattering band, this was near the bottom of the gradient and corresponded to the bottom component of the virus. This has a sedimentation coefficient of 126*S* (Stace-Smith, 1970, 1985, Fig. 5.9).

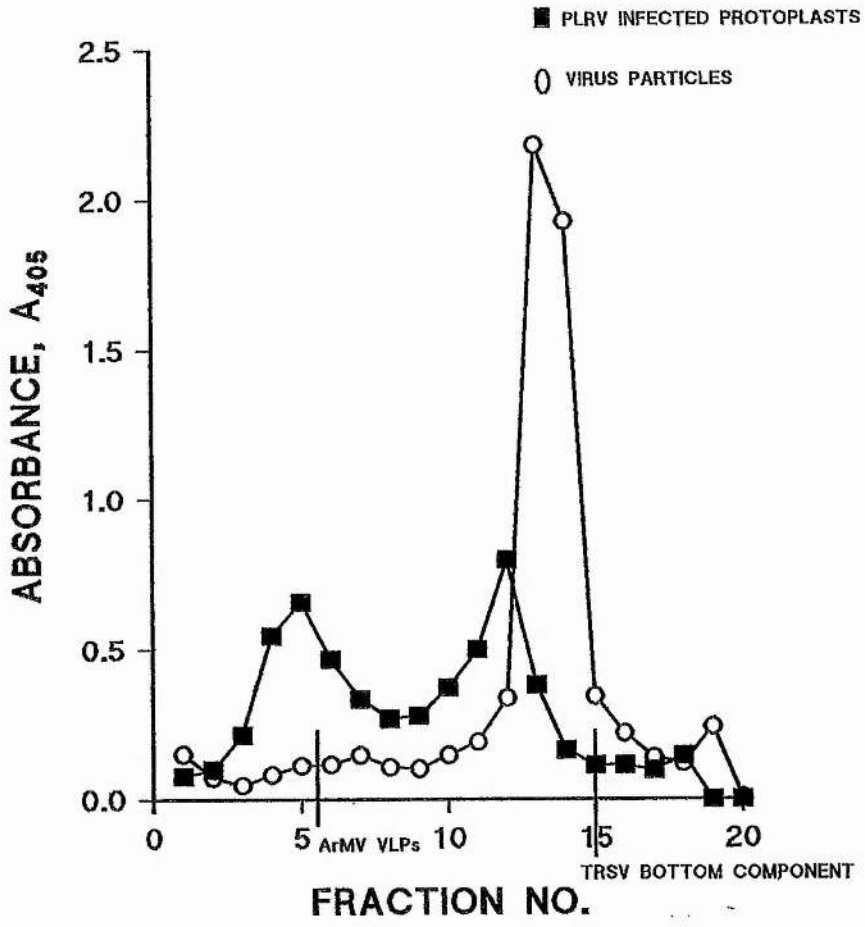
The ArMV top component preparation sedimented to a position near the top of the gradient. This component has a sedimentation coefficient of 53*S*. (Murant, 1970, Fig. 5.9).

Using these two values to calibrate the gradient, an approximate value was calculated for the sedimentation coefficient of the extra component found in lysates of PLRV-infected protoplasts. This value was estimated to be approximately 48*S*. Taking into account probable differences in sedimentation behaviour between PLRV and the two nepoviruses discussed, this number may be rather inaccurate, but it provides an approximate value which suggests that the contents of this component do not consist of either empty particles or particles containing subgenomic RNA only. Both of these molecules would sediment considerably lower in the gradient than the molecule detected by the ELISA test. The BWYV top component had a sedimentation coefficient of 62*S* (Hewings and D'Arcy, 1986) and using the calibration results for calculations, empty PLRV particles would have a sedimentation coefficient of 69*S*.

These results suggest that the top component of PLRV is probably not made up of complete protein shells, empty or otherwise. This reinforces the idea that some kind of RNA/protein association may be occurring since it

FIGURE 5.9: The calibration of sucrose gradients with ArMV and TRSV virus particles.

Particles of TRSV were purified from infected plants. Virus-like particles of ArMV were purified from *N. tabacum* plants which had been transformed with the ArMV coat protein gene as described by Bertioli *et al.* (1991). The particles were sedimented through sucrose gradients and their location determined by the observation of light scattering bands in the gradient. For TRSV, only bottom component particles were present in sufficient quantity to be located in this manner.


PLRV INFECTED PROTOPLASTS
VIRUS PARTICLES
2.5
2.0
1.5
1.0
0.5
0.0
ABSORBANCE, A405
0
5
ArMV VLPs
10
15
20
TRSV BOTTOM COMPONENT
FRACTION NO.

has already been established that subgenomic RNA and coat protein are both present in the same fraction.

6. DISCUSSION

The genomes of several isolates of PLRV have been sequenced; Scottish (Mayo *et al.*, 1989), Dutch (van der Wilk *et al.*, 1989), Australian and Canadian (Keese *et al.*, 1990). The subgenomic RNA was first reported by Mayo *et al.* (1984) and it was subsequently reported by Tacke *et al.* (1990) to be 2.3 kb in size with the 5'-end at position 3653 on the genome. Smith and Harris (1990) detected a subgenomic RNA of size 2.5 kb by Northern blot analysis of RNA extracted from tissue infected with an American isolate of the virus.

The subgenomic RNA of the PLRV isolate described in this report was established to be 2.5 kb in size, mapping to position 3376 on the genome (using the sequence of the Dutch isolate, van der Wilk *et al.*, 1989). Therefore the subgenomic RNA of the Scottish isolate is 177 nucleotides longer than that of the German isolate. As described previously, the 5'-end of the Scottish isolate subgenomic RNA matches exactly the first eight nucleotides of the 5'-end of the genomic RNA and this feature has also been noted for BWYV (V. Zeigler-Graf, personal communication), and in viruses of other groups. However, when the subgenomic RNA of BYDV-PAV was characterised, no such sequence similarity was found although a repeat was found upstream of the 5'-end. PLRV and BYDV-PAV are in different subgroups of the luteovirus group and have several genomic traits which distinguish them. This further distinction may add to those which already

exist, perhaps reflecting differences in the polymerase genes of viruses in the two subgroups.

6.1 SUBGENOMIC RNA PROMOTERS

The subgenomic RNA promoters of some plant viruses have been characterised quite thoroughly. French *et al.* (1986) established that the promoter of the subgenomic RNA of BMV stretched no more than 17 bases downstream of the intiation site and French and Ahlquist (1988) reported that it extended up to 95 bases upstream. An oligo [A] tract and a repeated sequence were proposed to be important functional elements.

Further work proposed that the complete subgenomic promoter of BMV encompassed 62 bases and comprised 4 functional domains; a core sequence, a poly-[A] tract, an upstream UUAUUAUU block and a downstream A-U tract (Marsh *et al.*, 1988). Deletions of parts of this promoter had different down- regulating effects on the synthesis of the subgenomic RNA. Within the core sequence were 4 sequence blocks with homology to either the postulated alphavirus promoter sequences or the intercistronic regions of other plant viruses and these were upstream of the start of the 5'-end of the subgenomic RNA. Evidence of homologies between sequences at the 5'-termini of the genomic RNAs and the subgenomic promoter was suggested to be significant in the overall replication strategy of BMV and probably other plant viruses.

Structural motifs have also been reported to be involved in subgenomic RNA promoters (Goulden *et al.*, 1990) where the sequence GCAUA has been reported to be important in the generation of subgenomic RNAs of isolates of TRV, the internal A being the first residue of the messenger RNA (Goulden *et al.*, 1990). This residue is contained within a region, found in all tobraviral RNA 2 sequences examined, which is potentially able to form a nine base pair stem structure with a terminal loop of 4 nucleotides. This appeared to be true for ORFs, such as the 16K ORF (Angenent *et al.*, 1989a, 1989b), which are expressed by the production of subgenomic RNAs but similar motifs were not found upstream of other tobraviral ORFs. However, the constancy of this structure, in terms of its location and content, suggests that it is significant and has a role in promoting subgenomic RNA synthesis (Goulden *et al.*, 1990).

Computer-generated secondary structures of BMV subgenomic RNA 4 revealed a stable hairpin at the 5'-terminus, the initiation site of RNA 4 being located at the start of the loop. This was shown to form when as few as 20 bases upstream of the initiation site were present and was proposed to be part of the recognition signal for the initiation of the subgenomic RNA synthesis (Miller *et al.*, 1985)

Similar computer studies were conducted for PLRV in the region between nucleotides 3095 and 3625, the sequence surrounding the proposed initiation site of the subgenomic RNA, but no significant, stable hairpin or pseudoknot structures were observed. Moreover, of the sequences described to make up the promoter of BMV RNA 4 (Marsh *et al.*, 1988), none were

found upstream of the start of the subgenomic RNA of PLRV. The poly[A] tract was completely absent and there was apparently nothing upstream of the start site which resembled the UUAUUAUU block described by Marsh *et al.* (1988). However, there were two U-A rich regions downstream of the proposed 5'-end starting at positions 3465 and 3538 (as according to van der Wilk *et al.*, 1989) although each of these 10 nucleotide sequences differed by one base between the Scottish (Mayo *et al.*, 1989) and Dutch sequences, both changes being A-U transversions. This single base change probably does not result in a significant difference in the function of the sequence, if it is functional. The AAGA sequence suggested to be part of the core sequence of the BMV subgenomic promoter was also present twice in the untranslated leader sequence of the PLRV subgenomic RNA. One of these was at the extreme 5'-end and was part of the described direct repeat sequence at the genomic and subgenomic 5'-ends and the second was at a position 61 nucleotides before the start of the coat protein gene. In BMV RNA, this sequence motif was also upstream of the initiation site of RNA 4. A possible subset of another part of the core sequence was observed, CCUAA was located directly before the second AAGA sequence and lacked the first two nucleotides (GU) of the sequence as described by Marsh *et al.* (1988).

The sequence GCAUA suggested by Goulden *et al.* (1990) to be important in the formation of the subgenomic RNA of TRV was found quite early in the untranslated 5' leader sequence (at position 39) in the subgenomic RNA of PLRV. This sequence was only one nucleotide different from one of the core sequences proposed by Marsh *et al.* (1988) (GCGUA)

for BMV, in both cases the messenger starting on the third nucleotide of the sequence.

The location of each part of the proposed promoter sequence is also reported to be important (Marsh *et al.*, 1988). For BMV, the poly[A] tract was proposed to serve as an unstructured spacer (Marsh *et al.*, 1988) and Ahlquist *et al.* (1981) suggested that it formed part of the stable hairpin structure. Furthermore, French and Ahlquist (1987) found that its presence also appeared to promote accumulation of RNA 3 (from which the subgenomic RNA is tanscribed).

Marsh *et al.* (1988) also suggested that the upstream UUAUUAUU block together with the poly[A] tract was not required for the correct initiation of subgenomic RNA transcription but did affect the quantity produced. However, neither the sequences nor the hairpin structure are present at or near this location in the PLRV genome. Moreover, of the core sequences of the BMV RNA 4 promoter which have been observed in PLRV RNA, namely the AAGA sequence, part of the GUCCUAA sequence and the downstream (A-U) tract, only one, the (A-U) tract, was in the suggested location, and there was no sign of promoter sequences present upstream of the 5'-end of the subgenomc RNA. However, in addition to the 5'-end sequence homologies between subgenomic and genomic RNAs described earlier, French and Ahlquist (1987) noted that a sequence element was conserved between the intercistronic sequence of BMV RNA 3 from which the subgenomic RNA arises, and the 5'-non-coding sequences of BMV RNAs 1 and 2.

A lengthy 5'-untranslated leader sequence, like that of the PLRV subgenomic RNA, appears not to be required by BMV RNA 4, perhaps suggesting that it is unnecessary for the stability of this molecule. Another explanation is that such a sequence, if functional, may not be required in close linkage to the RNA 4 initiation site to direct subgenomic RNA synthesis (French and Ahlquist, 1987) whereas in PLRV it may be an absolute requirement. A further idea connected with this observation is that BMV RNA 4 is encapsidated (Kaesberg, 1987) whereas PLRV subgenomic RNA is not. Encapsidation may not allow for the RNA 4 to carry any sequence other than that which is essential for coding, replication and stability of the molecule. The sequence of the PLRV subgenomic RNA is not affected by spatial limitations and the untranslated leader sequence of PLRV subgenomic RNA may provide the coding sequences with some added protection.

Lehto *et al.* (1990) postulated that the promoter/leader sequences in front of the ORFs of TMV determine the timing of gene expression. There appears to be no sequence similarity between the putative RNA-promoter regions for the two subgenomic RNAs of TMV suggesting that they might be regulated independently. It is suggested that different trans-acting factors may be involved in promoter recognition to determine when a gene is expressed. Alternatively, different replicase functions may recognise the different subgenomic RNA promoters (Lehto *et al.*, 1990).

This suggests that the 5'-untranslated leader sequence of the PLRV subgenomic RNA has more than one function. It may be used to promote

transcription of the RNA and also translation of the coat protein. If so, factors encoded by the genomic RNA may be involved in the recognition of one or more sequences on the subgenomic RNA which would subsequently promote transcription and coat protein synthesis.

AMV has been reported to have a subgenomic RNA which is involved in the expression of the coat protein gene and the promoter for this RNA was observed to stretch for more than 53 nucleotides downstream of the subgenomic RNA 4 initiation site (van der Kuyl *et al.*, 1991). Moreover, the promoter of this RNA, which is upstream of the initiation site, is also reported to overlap with the preceeding cistron (van der Kuyl *et al.*, 1990). The 5'-untranslated region of the PLRV subgenomic RNA starts within the 3'-end of the putative polymerase gene (Mayo *et al.*,1989) and presumably the length of this region holds some implications for which genes are expressed, when and to what level. Some of the features described as promoter sequences in other plant viruses (e.g. TRV, Goulden *et al.*, 1990; BMV, Marsh *et al.*, 1988) have also been found in the untranslated leader sequence of the subgenomic RNA of PLRV but in each case, the sequence described is in a different location from that reported for the original virus. It is unlikely that a sequence which formed an important part of the promoter core in BMV would be important in PLRV if located in a completely different position and surrounded by different and apparently unrelated sequence elements.

The sequence repeat which was observed at the 5'-ends of the PLRV genomic and subgenomic RNAs has not been reported to play an important

role in the subgenomic RNA promoters characterised to date, although Marsh *et al.* (1988) stated that for BMV, homologies between sequences at the 5'-termini of the genomic RNAs and the subgenomic promoters were likely to be significant in the overall replication strategy. Several viruses have been shown to have a direct repeat of the 5'-terminal genomic sequence at or near the 5'-terminus of the subgenomic RNA and these include AMV (Symons, 1985) and TRV (Cornelissen *et al.*, 1986), two viruses which have been studied extensively. Cornelissen *et al.* (1986) proposed that the 5'-terminal sequence which was identical in RNA1 and RNA2 of TRV strain PSG may reflect part of a replicase recognition signal in the corresponding minus-strand RNAs and the 5'-terminal sequence repeat found in the subgenomic RNA may reflect part of an internal initiation site for the replicase in minus-strand RNA 2.

The 5'-terminal sequences of the genomic RNA of PLRV is almost identical to that of BWVY (Keese *et al.*, 1990) and there are very similar sequences about 3473 nucleotides from the 5'-ends of the genomic RNA of both viruses (Mayo *et al.*, 1989). However, there are few substantial similarities in sequences upstream of this point. If the mechanism of generation of PLRV subgenomic RNA involves internal initiation on minus-strand templates, as has been shown for BMV (Marsh *et al.*, 1988), then perhaps any upstream promoter sequences are specific to each virus whereas the factors recognising downstream promoter sequences are less specific. However, this is not so for viruses such as BMV and TRV (Marsh *et al.*, 1988; Goulden *et al.*, 1990) in which the putative promoter sequences

apparently shared with other viruses are upstream rather than downstream of the start of the subgenomic RNA. By contrast, it may be true for AMV which has a subgenomic promoter stretching 53 nucleotides downstream from the 5'-end (van der Kuyl *et al.*, 1991) and for beet necrotic yellow vein virus (BNYVV) where the subgenomic promoter domain is situated downstream rather than upstream of the transcrpition initiation site (Balmori *et al.*, 1993).

Another factor to consider is that TRV and BMV are bi- and tripartite respectively, whereas PLRV is monopartite. The absence of trans-acting factors effective in the promotion of transcription of the subgenomic RNA may result in the development of a less defined promoter sequence than that of BMV. Moreover, as mentioned earlier, the untranslated leader sequence may contain promoter regions which have a function in coat protein synthesis, readthrough protein synthesis and P4 synthesis. Since there is no evidence of a second subgenomic RNA being involved in PLRV replication, the 5'-leader sequence of this molecule would have to contain all the information necessary for these functions. Furthermore, as for TMV (Lehto *et al.*, 1990), it is not necessarily true that the sequence needed to promote or regulate the synthesis of these proteins is the same in each case.

Although some consensus sequences found in promoters of subgenomic RNAs in other viruses were also found in the 5'-leader sequence of the subgenomic RNA of PLRV, their location and content suggest that they probably do not fulfill the same role. In order to assess the extent of subgenomic promoters, their effects and possible core and flanking sequences for PLRV RNA, deletion analysis such as that described by Goulden *et al.*

(1990) and Marsh *et al.* (1988) should be performed. Results may indicate the true importance of 5'-end sequence repeats and of other internal sequences which have been found in the subgenomic RNA promoters of other viruses.

6.2 VIRUS REPLICATION IN PROTOPLASTS

Previously, PLRV had been shown to infect protoplasts isolated from both tobacco and potato plants (Barker and Harrison, 1982; Kubo and Takanami, 1979) and I have reported that it also infects protoplasts isolated from *Nicotiana clevelandii* and *Chenopodium quinoa.* Infection of *C. quinoa* protoplasts was rather poor, perhaps due to the source plant being an inappropriate host for PLRV replication. The low infection rate made detailed studies impossible. In the other protoplasts used, namely *N. tabacum* cv. Xanthi (transgenic and wildtype), *N. tabacum* cv. Samsun (transgenic and wildtype) and *N. clevelandii*, the results of infection seemed not to differ significantly. Northern blotting showed that both RNA species were present in large quantities at the expected locations. The protoplasts which were isolated from the transgenic plants [*N. tabacum* cv. Xanthi transformed with the coat protein gene of ArMV (Bertioli *et al.*, 1991) and *N. tabacum* cv. Samsun transformed with the coat protein gene of PLRV (Barker *et al.*, 1992)] appeared to show no resistance to PLRV infection and multiplication. In contrast to this result, Barker *et al.* (1992) demonstrated that intact potato

plants transformed with the PLRV coat protein construct were resistant to PLRV multiplication.

The growth curve of PLRV accumulation in protoplasts isolated from *N. tabacum* cv. Xanthi was similar to that of TMV and TRV accumulation in tobacco protoplasts (Huber *et al.*, 1977; Harrison *et al.*, 1976). Harrison *et al.* (1976) reported that for TRV, infective RNA was detected after 7 hrs of infection and nucleoprotein particles after 9 hrs. It was proposed that coat protein was not accumulated before these particles were produced. Infective RNA synthesis was reported to be complete at 12 hrs but its incorporation into nucleoprotein was not complete until 24 hrs or longer post-inoculation.

For PLRV, however, neither RNA nor protein could be detected until approximately 16 hrs after inoculation. Subgenomic RNA must be transcribed before the coat protein can be translated and this may result in later accumulation of particle protein. For this reason, one would expect to be able to detect the RNA at a relatively early stage in infection. The early samples taken at 3 and 7 hrs after inoculation did not appear to contain any PLRV RNA but RNA was observed 15 hrs after infection (results not shown). This indicated that a high level of replication had taken place between 7 and 15 hrs post-inoculation and yielded enough genomic and subgenomic RNA to be detected on a Northern blot. Since the subgenomic RNA was now also present, coat protein synthesis probably also started between 7 and 15 hrs after inoculation.

The quantity of PLRV RNA present in the protoplasts appeared to reach a peak at approximately 48 hrs after infection and did not increase

further. Whether this RNA was newly synthesised or had been stored somewhere in the protoplast is impossible to tell from this assay, but since the level of production of virus particles appeared to reach a plateau also at about 48 hrs post-inoculation, this RNA was unlikely to be newly transcribed. If large quantities of fresh RNA was being made, protein and virus particles would also be made. It is unlikely that the protoplast could continue to support high levels of virus production.

Gill and Chong (1976) reported that in plants infected with BYDV subgroup 2 (containing BYDV strains RMV and RPV and thus similar in genome arrangement to BWYV and PLRV) fibrils were present in membranous vesicles in the cytoplasm and speculated that RNA may constitute these fibrils. This may be a method for non-encapsidated RNA (genomic and subgenomic) to be stored in the cytoplasm without degradation. If true for protoplasts infected with PLRV, it may explain why large quantities of viral RNA appeared to be present when the actual virus multiplication rate was very low.

6.2.1 THE EFFECT OF TEMPERATURE ON VIRUS PRODUCTION IN PROTOPLASTS

An increase in temperature, from 20 to 25°C, in the incubation conditions of protoplasts appeared to increase the yield of PLRV significantly. Comparisons of PLRV-inoculated protoplasts incubated at the

two temperatures showed that after various time intervals, those incubated at 25°C contained more virus than did those which had been incubated at 20°C. It is possible that this temperature may be more similar to the internal temperature of the plant. However, protoplasts isolated from many other species of plant and inoculated with various viruses show best results at a range of temperatures which have presumably been selected for improved virus yield. The protoplasts, themselves, appeared to survive in larger numbers at the lower temperature.

6.2.2 THE EFFECT OF DIFFERENT LIGHT CONDITIONS ON VIRUS PRODUCTION IN PROTOPLASTS

The inclusion of a dark period in the incubation of PLRV-inoculated protoplasts increased the amount of virus production. This may have been for one of several reasons.

1. The protoplasts may have been preconditioned to light and dark cycles due to the growth conditions of the plants from which they were isolated. The plants were incubated for 16 hrs in the light and for 8 hrs in the dark. This treatment may have had an effect on the protoplasts, leaving them able to support virus multiplication more easily and to a higher level when a dark period was included during protoplast incubation.

It is possible that in virus-infected protoplasts, a high level of activity takes place during the light period which results in virus production and the

synthesis or accumulation of surplus nutrients and metabolites. The dark period allows the protoplasts some recovery whilst still supporting virus multiplication. In light only incubations, the protoplasts are not allowed this period of recovery and may therefore be unable to produce PLRV in as large quantities.

2. The protoplasts may contain large quantities of indigenous metabolites which are used during the dark periods to continue virus production and during the light period to increase virus production.

3. The dark period may act as a trigger, recognised by virus-coded factors to begin a mode of increased virus multiplication. The trigger may be the presence or absence of a product whose synthesis is induced by certain light conditions. For example, during conditions of strong light intensity, the protoplasts may produce a factor which represses virus multiplication to some extent. When the dark conditions begin, the repressing factor is removed or its synthesis stopped, allowing virus multiplication to proceed at its highest level.

Alternatively, the dark period may trigger the production of a factor which enhances multiplication. If this is not removed by the change to the light period, increased virus production will continue.

To test this hypothesis, comparative samples could be taken from protoplasts incubated in constant light and those incubated in light and dark cycles. The samples should be taken over a period of several days at the end of each light and dark period and the antigen content assayed by ELISA. This should determine whether virus multiplication proceeds at the same rate in

the two sets of samples, or if the first and subsequent dark periods cause the rate of multiplication to be consistently higher in the samples incubated in light and dark conditions as compared to those incubated in the light only conditions. This may help to determine under which conditions most virus multiplication takes place.

It was revealed that the yield of virus was higher in protoplasts which had been incubated solely in the dark compared to those which had been incubated solely in the light. This suggests that the initial increase in multiplication is completely dark-associated but may be further increased by the inclusion of light periods in the incubation conditions. It is possible that this is a feature of the virus or the protoplasts or a combination of both. It was reported by Reunova *et al.* (1992) that light conditions other than normal light at about 2000 lux resulted in the decreased yield of TMV in tobacco protoplasts, and this also appeared to be the case for TRSV (see Chapter 4). Light conditions other than normal light and total darkness have not been tested here but it would be interesting to determine which component of light, if any, is important in PLRV multiplication.

Kano *et al.* (1985) reported that light accelerated TMV production, although it was not essential. Moreover, it was suggested that photosynthesis, more specifically non-cyclic photosynthetic electron transport accompanied by ATP synthesis, played a major part in the promotion of TMV production by light. Experiments performed using various inhibitors of photosynthesis and respiration demonstrated that both of these processes are involved in TMV multiplication, although it appeared that the more stimulatory role

belonged to photosynthesis. This may suggest that photosynthesis does not have such an active role in PLRV production. Experiments using inhibitors such as those described by Kano *et al.* (1985) may help to clarify this.

It is interesting to note that the inclusion of a short period of light at the beginning of incubation appeared to give the highest virus yield in protoplasts. As the length of the light period increased during incubation, the production of virus decreased. However, a short period of darkness at the beginning of the incubation period also gave a high yield of virus in protoplasts. These results appear to be completely contradictory but they may suggest that it is the change in light conditions which triggers enhanced virus multiplication. It is possible that once the inoculated protoplasts have been incubated in the dark, even for a very short period, the required event has taken place and increased PLRV multiplication is the result, whether the protoplasts are left in the dark or returned to the light. Again, this event may be host- or virus-encoded or a combination of both and it may perhaps be linked to the phloem limitation of the virus in whole plants.

6.2.3 PRODUCTION OF THE PLRV P-5 PROTEIN IN TOBACCO PROTOPLASTS

Bahner *et al.* (1990) postulated that the ORF immediately downstream of the coat protein gene is translated by readthrough of the amber termination codon of the coat protein gene, thereby producing a protein comprising the

products of the coat protein ORF linked to the P-5 ORF. This protein was detected in protoplast extracts and estimated to have a molecular weight of 80K, and in purified virus particles where it was estimated to have a molecular weight of 53K. However, similar immunoblotting experiments performed here using a monoclonal antibody directed against the coat protein of the virus detected protein which corresponded to a molecule the size of the coat protein only. Since the readthrough protein is probably translated in only tiny quantities compared to the coat protein, detection may be difficult. However, a 53K polypeptide was easily detected in purified virus particles.

Immunoblotting done with a polyclonal antiserum (gift from Dr. J. Lamb) directed against the non-coat protein part of the postulated readthrough protein yielded several high molecular weight bands in both PLRV- and mock-infected protoplast extracts. No virus-specific bands were observed. Using this antiserum, Bahner *et al.* (1990) were able to detect polypeptides of 80K and 90K in PLRV-infected protoplasts but not in mock-infected protoplasts, and a polypeptide of 53K in purified virus particles. This band was also detected in purified particles by a MAb directed against the coat protein and it was proposed that part of the protein had been lost from the C-terminal end.

The inability to detect the larger protein in the protoplast extracts used here implies that it is an effect of multiplication in these protoplasts which has caused its disappearance or prevented its synthesis by reducing the amount of readthrough which has taken place. The conditions of protoplast incubation may have an effect, constant light perhaps directing the virus to

make coat protein in preference to readthrough protein. Nevertheless, the culture conditions used here were the same as those used by Bahner *et al.* (1990), allowing for slight differences in temperature and light intensity, as was the isolation procedure and subsequent electrophoresis and immunoblotting.

It is possible that slight differences in the stability or condition of the protoplasts or the virus inoculum may result in differential production of viral proteins but this type of variation is very difficult to assess or reproduce.

6.3 THE PRODUCTION OF A VIRAL COMPONENT OTHER THAN THAT OF VIRUS PARTICLES

An antigenic component other than whole virus particles was found in extracts of PLRV-infected protoplasts. This was observed to sediment to a position near the top of a sucrose gradient and to contain coat protein and subgenomic RNA. Calibration of sucrose gradients with ArMV empty particles showed that the PLRV top component sedimented too slowly for it to comprise subgenomic RNA encapsidated in an approximately 30 nm diameter virion, and EM could detect no such structures in the fraction containing this component. The extra component was not present in purified preparations of virus particles, nor was it present in extracts of infected potato tissue. Both of these findings suggest that the molecule found in

protoplast extracts was probably rather unstable and may be an intermediate in the formation of new, infectious virus particles.

The top components which have been characterised in other viruses such as TRSV and ArMV have generally consisted of empty coat protein shells (Stace-Smith, 1970; Murant, 1970). BWYV and BYDV have also been reported to make empty virions (Hewings and D'Arcy, 1986; Proll *et al.*, 1985), having sedimentation coefficients of 62*S* and 53*S* respectively.

For BWYV, these structures were derived from partially purified virus particles which had been retrieved from a rate-zonal density gradient. PLRV virus particles purified as described by Harrison (1984) did not include similar structures, and only a single component was found after density gradient centrifugation (Harrison, 1984). Hewings and D'Arcy (1986) reported that nucleic acid was not associated with the top component of their BWYV isolate but it is possible that the quantity of material used for electrophoresis was too small for RNA to be visualised in the ethidium bromide stained gel used. It is possible that virions of PLRV which contain only subgenomic RNA do exist *in vivo* but are low in number and easily destroyed by treatments such as gradient centrifugation and virus purification. However, if so, the remains of these structures have been found where the coat protein and the nucleic acid are still associated with each other.

However, questions arise as to how the subgenomic RNA became encapsidated initially. The subgenomic RNA is not encapsidated with genomic RNA. This may be due to one of several reasons: the subgenomic RNA may lack the required sequences for encapsidation; there may be too

little room inside the virion for both RNA species to be encapsidated or the genomic RNA may be encapsidated prefentially, although the subgenomic RNA may also have sequences which allow encapsidation. If the required encapsidation sequences were lacking, the subgenomic RNA would not be encapsidated into true virions, even without the genomic RNA. Spatial requirements within the virion would prevent two RNA species from being encapsidated together, but if the subgenomic RNA only was to be encapsidated, one would predict that the virus particle would be unstable. This indeed may be the case if the top component of PLRV is in the form of partially filled virus particles which have been denatured during gradient centrifugation. However, as mentioned earlier, sedimentation of the top component indicated a molecule less dense than a virus particle containing the subgenomic RNA. Degradation may have caused the loss of some of the protein making up the virion or shells made up of a different protein subunit configuration may be involved. Bancroft (1970) postulated that cowpea chlorotic mottle virus (CCMV) RNA had a structural role in the virion. This may be a common feature in icosahedral viruses and in the case of PLRV, the subgenomic RNA and coat protein may have associated to form a sub-particle structure. This may be involved in particle assembly, allowing the subgenomic RNA to remain intact for periods of time in order to continue the synthesis of coat protein.

6.4 CONCLUSIONS

This thesis has attempted to establish a groundwork for PLRV multiplication in protoplasts and, to some extent, in plants. For viruses such as PLRV, where manual inoculation is impossible and the alternatives are difficult to manipulate successfully, protoplasts have proven to be an excellent system for use in studies of virus multiplication.

Here, the timing and location of virus multiplication have been examined, taking into account the effect of protoplast incubation conditions. In some instances, such as the basic time course of virus multiplication, results previously seen with other viruses such as TMV (Huber *et al.*, 1977) and TRV (Harrison *et al.*, 1976) were also observed here. However, in other experiments, such as the effects of dark conditions on PLRV multiplication, unexpected results were obtained which, in the long run, may help understanding of PLRV multiplication in the phloem tissue of plants. Nevertheless, further work is required before definite conclusions can be drawn.

The 5'-end of the PLRV subgenomic RNA has been mapped and a potential recognition site for the virus replicase revealed on the genomic RNA. The leader sequence of the subgenomic RNA of PLRV may prove to be multifunctional, containing promoters for the production of both the subgenomic RNA and proteins which are proposed to be expressed by this RNA. Characterisation of these promoters would be a large step forward in the unravelling of PLRV multiplication.

A possible sub-particle structure has also been found which apparently consists of subgenomic RNA and coat protein. This could not be visualised using EM and its possible function could only be surmised although the immediate view is that the coat protein is protecting the subgenomic RNA from degradation.

As more information becomes available about PLRV and luteoviruses in general, the complex nature of these viruses becomes more and more apparent. It is hoped that with improved knowledge of the multiplication of these viruses, information may be acquired which will allow plant virologists to render plants resistant to infection by luteoviruses. Indeed, some success has already been achieved in this direction with PLRV (Barker *et al.*, 1992). It is hoped that, with its details of PLRV multiplication, this thesis may aid future developments in luteovirus research and the creation of luteovirus resistant plants.

SUMMARY

1. PLRV produces one subgenomic RNA during multiplication. Probing studies and primer extension analysis determined its size to be 2.5 kb. It was shown not to be encapsidated.

2. A sequence was found at the 5'-end of the genomic RNA which was directly repeated at the proposed 5'-end of the subgenomic RNA. This sequence may form part of an internal recognition signal on the negative strand for the replicase complex.

3. Migration of the subgenomic RNA was constant, regardless of its origin.

4. The subgenomic RNA was detected in extracts of all species of protoplasts inoculated with PLRV, including those isolated from tobacco plants transformed with the PLRV coat protein gene.

5. The subgenomic RNA was detected in extracts of most species of PLRV-infected plant. Viral RNA was not detected in extracts of PLRV-infected tobacco plants which had been transformed with the PLRV coat protein gene. Subgenomic RNA was not detected in PLRV-infected plants of the transgenic line of potato, B3. This was thought to indicate that although the plants were infected, multiplication was not taking place.

6. There was no difference between the PLRV RNA on northern blots of the three different isolates tested; Scottish, 11 and 30.

7. The stem of PLRV-infected potato plants contained the most PLRV-specific RNA compared to tissue taken from other parts of the plant. PLRV RNA detected in extracts taken from young plants was less denatured than that taken from older plants.

8. A virus concentration of 0.1 μg/ml was found to be the optimal for PLRV inoculation of tobacco protoplasts.

9. In protoplasts, more PLRV accumulation took place at 25°C than at 20°C.

10. In protoplasts, the majority of PLRV multiplication took place in the first 50 hrs after inoculation. RNA was detected as early as 15 hrs post-inoculation but coat protein could not be detected until after 24 hrs post-inoculation.

11. The inclusion of a dark period during the incubation of PLRV-infected protoplasts increased the amount of PLRV accumulation. No such effect was observed with TRSV-infected protoplasts.

12. The inclusion of a dark period in the incubation conditions of PLRV-infected protoplasts appeared to decrease the quantity of virus-specific RNA

in the protoplasts.

13. PLRV-infected protoplasts incubated in continuous dark contained more virus than PLRV-infected protoplasts incubated in continuous light. The inclusion of 2% sucrose in the incubation medium did not increase the yield of virus in either light or dark conditions.

14. In extracts of PLRV-infected protoplasts, most of the virus particles and virus RNA present sedimented at 2000 rpm. The larger cellular organelles such as the chloroplasts also sedimented at this speed. RNA extracts of the supernate remaining from high speed centrifugation contained only genomic RNA suggesting that some virus particles were present which were not associated with any organelle or membranous structure.

15. A component which sedimented more slowly than the main component of 115*S*, was found when lysates of PLRV-infected protoplasts were subjected to sucrose gradient centrifugation. No visible structures associated with this component were observed using the electron microscope and subgenomic RNA, only, was detected by Northern blotting.

16. The extra component was not detected in tissue extracted from PLRV-infected potato.

17. Treatment with paraformaldehyde caused the virus particles to sediment

more quickly than untreated virus particles.

18. Calibration of the profiles of the sucrose gradients with ArMV virus-like particles and TRSV bottom component indicated that the extra component found in lysates of PLRV-infected protoplasts probably sedimented more slowly than predicted for PLRV-like particles which lack RNA.

REFERENCES

ABEL, P.P., NELSON, R.S., DE, B., HOFFMANN, N., ROGERS, S.G., FRALEY, R.T. & BEACHY, R.N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science*, **232**, 738-743.

ACOSTA, O. & MAYO, M.A. (1990). Accumulation of different types of raspberry ringspot nepovirus particle in infected *Nicotiana* protoplasts. *Journal of General Virology*, **71**, 713-717.

ADAMS, A.N. & HULL, R. (1972). Tobacco yellow vein, a virus dependent on assistor viruses for its transmission by aphids. *Annals of Applied Biology*, **71**, 135-140.

AHLQUIST, P., LUCKOW, V. & KAESBERG, P. (1981). Complete nucleotide sequence of brome mosaic virus RNA3. *Journal of Molecular Biology*, **153**, 23-38.

ALBLAS, F. & BOL, J.F. (1977). Factors influencing the infection of cowpea mesophyll protoplasts by alfalfa mosaic virus. *Journal of General Virology*, **36**, 175-185.

ALBLAS, F. & BOL, J.F. (1978). Coat protein is required for infection of cowpea protoplasts with alfalfa mosaic virus. *Journal of General Virology*, **41**, 653-656.

ANGENENT, G.C., POSTHUMUS, E. & BOL, J.F. (1989a). Biological activity of transcripts synthesised *in vitro* from full-length and mutated DNA copies of tobacco rattle virus RNA-2. *Virology*, **173**, 68-76.

ANGENENT, G.C., VERBEEK, H.B.M. & BOL, J.F.(1989b). Expression of the 16K cistron of tobacco rattle virus in protoplasts. *Virology*, **169**, 305-311.

AOKI, S. & TAKEBE, I. (1969). Infection of tobacco mesophyll protoplasts by tobacco mosaic virus ribonucleic acid. *Virology*, **39**, 439-448.

APPEL, O. (1906). Neuere Untersuchungen uber Kartoffel und Tomatenerkrankungen. *Jahresbericht der Vereinigung fur Angewandte Botanik*, **3**, 130-136.

ASHBY, J.W. (1984). Bean leafroll virus. *CMI/AAB Descriptions of Plant Viruses*, no. 286.

BAHNER, I., LAMB, J., MAYO, M.A. & HAY, R.T. (1990). Expression of the genome of potato leafroll virus: readthrough of the coat protein termination codon *in vivo*. *Journal of General Virology*, **71**, 2251-2256.

BAINS, P.S., PAPPU, H.R. & HIRUKI, C. (1988). An improved system for clover yellow mosaic virus infection of pea leaf mesophyll protoplasts. *Annals of the Phytopathological Society of Japan*, **54**, 174-182.

BAJET, N.B. & GOODMAN, R.M. (1981). Infection of bean mesophyll protoplasts by bean golden mosaic virus DNA. *Phytopathology*, **71**, 201.

BALMORI, E., GILMER, D., RICHARDS, K., GUILLEY, H. & JONARD, G. (1993). Mapping the promoter for subgenomic RNA synthesis on beet necrotic yellow vein virus RNA-3. *Journal of General Virology*, in press.

BANCROFT, J.H. (1970). The self-assembly of spherical plant viruses. *Advances in Virus Research*, **16**, 99-134.

BARKER, H. (1988). Potato leafroll. Unpublished review.

BARKER, H. & HARRISON, B.D. (1977a). Infection of tobacco mesophyll protoplasts with raspberry ringspot virus alone and together with tobacco rattle virus. *Journal of General Virology*, **35**, 125-133.

BARKER, H. & HARRISON, B.D. (1977b). The interaction between raspberry ringspot and tobacco rattle viruses in doubly-infected protoplasts. *Journal of General Virology*, **35**, 135-148.

BARKER, H. & HARRISON, B.D. (1978). Double infection, interference and superinfection in protoplasts exposed to two strains of raspberry ringspot virus. *Journal of General Virology*, **40**, 647-658.

BARKER, H. & HARRISON, B.D. (1982). Infection of potato mesophyll protoplasts with five plant viruses. *Plant Cell Reports*, **1**, 247-249.

BARKER, H. & HARRISON, B.D. (1985). Restricted multiplication of potato leafroll virus in resistant potato genotypes. *Annals of Applied Biology*, **107**, 205-212.

BARKER, H., REAVEY, B., KUMAR, A., WEBSTER, K.D. & MAYO, M.A. (1992). Restricted multiplication in potatoes transformed with the coat protein gene of potato leafroll luteovirus: similarities with a type of host gene-mediated resistance. *Annals of Applied Biology*, **120**, 55-64.

BARKER, J.M., M^{c}INNES, J.L., MURPHY, P.J. & SYMONS, R.H. (1985). Dot-blot procedure with ^{32}P-DNA probes for the sensitive detection of

avocado sunblotch and other viroids in plants. *Journal of Virological Methods*, **10**, 87-98.

BARNETT, A., HAMMOND, J. & LISTER, R.M. (1981). Limited infection of cereal leaf protoplasts by barley yellow dwarf virus. *Journal of General Virology*, **57**, 397-401.

BEACHY, R.N., LOESCH-FRIES, S., & TUMER, N.E. (1990). Coat protein mediated resistance against virus infection. *Annual Review of Phytopathology*, **28**, 451-474.

BEIER, H. & BRUENING, G. (1975). The use of an abrasive in the isolation of cowpea leaf protoplasts which support the multiplication of cowpea mosaic virus. *Virology*, **64**, 272-276.

BEIER, H. & BRUENING, G. (1976). Factors influencing the infection of cowpea protoplasts by cowpea mosaic virus RNA. *Virology*, **72**, 363-369.

BEIER, H., ISSINGER, O.G., DEUSCHLE, M. & MUNDRY, K.W. (1981). Translation of the RNA of cowpea severe mosaic virus *in vitro* and in cowpea protoplasts. *Journal of General Virology*, **54**, 379-390.

BERTIOLI, D.J., HARRIS, R.D., EDWARDS, M.L., COOPER, J.I. & HAWES, W.S. (1991). Transgenic plants and insect cells expressing the coat protein of arabis mosaic virus produce empty virus-like particles. *Journal of General Virology*, **72**, 1801-1809.

BOCK, K.R. (1974). Maize streak virus. *CMI/AAB Descriptions of Plant Viruses*, no. 133.

BOCK, K.R. & HARRISON, B.D. (1985). African cassava mosaic virus. *CMI/AAB Descriptions of Plant Viruses*, no. 297.

BRAKKE, M.K. & ROCHOW, W.F. (1974). Ribonucleic acid of barley yellow dwarf virus. *Virology*, **61**, 240-248.

BROUGH, C.L., SUNTER, G., GARDINER, W.E. & BISARO, D.M. (1992). Kinetics of tomato golden mosaic virus DNA replication and coat protein promoter activity in *Nicotiana tabacum* protoplasts. *Virology*, **187**, 1-9.

BROWN, L.M. & WOOD, K.R. (1987). Translation of clover yellow mosaic RNA in pea mesophyll protoplasts and rabbit reticulocyte lysate. *Journal of General Virology*, **68**, 1773-1778.

BRUENING, G., BEACHY, R.N., SCALLA, R. & ZAITLIN, M. (1976). *In vitro* and *in vivo* translation of the ribonucleic acids of a cowpea strain of tobacco mosaic virus. *Virology*, **71**, 498-517.

BUCK, K.W. & COUTTS, R.H.A. (1985). Tomato golden mosaic virus. *CMI/AAB Descriptions of Plant Viruses*, no. 303.

CASPER, R. (1977). Detection of potato leafroll virus in potato and in *Physalis floridana* by enzyme-linked immunosorbent assay (ELISA). *Phytopathologische Zeitschrift*, **90**, 364-368.

CASPER, R. (1988). Luteoviruses. In *The Plant Viruses; Polyhedral Virions with Monopartite RNA Genomes*. Vol 3. pp. 235-258. Ed. R. Koenig. Plenum Press, New York.

CHIU, B.S. & TIEN, P. (1982). Infection of barley protoplasts with barley stripe mosaic virus detected and assayed by immunoperoxidase. *Journal of General Virology*, **58**, 323-327.

CLARK, M.F. & ADAMS, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, **34**, 475-483.

COCKBAIN, A.J. (1977). Bean yellow vein-banding virus. *Annual Report of the Rothamsted Experimental Station, 1977*, p. 221.

COCKBAIN, A.J., JONES, P. & WOODS, R.D. (1986). Transmission characteristics and some other properties of bean yellow vein-banding virus, and its association with pea enation mosaic virus. *Annals of Applied Biology*, **108**, 59-69.

CORNELISSEN, B.J.C., LINTHORST, H.J.M., BREDERODE, F.Th. & BOL, J.F. (1986). Analysis of the genome structure of tobacco rattle virus strain PSG. *Nucleic Acids Research*, **14**, 2157-2169.

COUTTS, R.H.A. & WOOD, K.R. (1976a). The infection of cucumber mesophyll protoplasts with tobacco mosaic virus. *Archives of Virology*, **52**, 59-69.

COUTTS, R.H.A. & WOOD, K.R. (1976b). Investigations on the infection of cucumber mesophyll protoplasts with cucumber mosaic virus. *Archives of Virology*, **52**, 307-313.

CREAMER, R. & FALK, B.W. (1990). Direct detection of transcapsidated barley yellow dwarf luteoviruses in doubly infected plants. *Journal of General Virology*, **71**, 211-217.

DAMSTEEGT, V.D. & HEWINGS, A.D. (1987). Relationships between *Aulacorthum solani* and soybean dwarf virus: effect of temperature on transmission. *Phytopathology*, **77**, 515-518.

D'ARCY, C.J. & DE ZOETEN, G.A. (1979). Beet western yellows virus in phloem tissue of *Thlaspi arvense*. *Phytopathology*, **69**, 1194-1198.

DAVIES, J.W. & HULL, R. (1982). Genome expression of plant positive-strand RNA viruses. *Journal of General Virology*, **61**, 1-14.

DAWSON, J.R.O., DICKERSON, P.E., KING, J.M., SAKAI, F., TRIM, A.R.H. & WATTS, J.W. (1978). Improved methods for infection of plant protoplasts with ribonucleic acid. *Zeitschrift fur Naturforschung*, **33C**, 548-551.

DE VARENNES, A., RUSSO, M. & MAULE, A.J. (1984). Infection of protoplasts from *Chenopodium quinoa* with cowpea mosaic and cymbidium ringspot viruses. *Journal of General Virology*, **65**, 1851-1855.

DIJKSTRA, J., VAN BEEK, N.A.M., LOHUIS, D., VAN HELDEN, M. & MEIJER, R. (1987). Is a helper factor necessary for infection of cowpea protoplasts with blackeye cowpea mosaic virus? *Netherlands Journal of Plant Pathology*, **93**, 43-47.

DINESH-KUMAR, S.P., BRAULT, V. & MILLER, W.A. (1992). Mapping and *in vitro* translation of a trifunctional subgenomic RNA of barley yellow dwarf virus. *Virology*, **187**, 711-722.

DOMIER, L.L., FRANKLIN, K.M., HUNT, A.G., RHOADS, R.E. & SHAW, J.G. (1989). Infectious *in vitro* transcripts from cloned cDNA of a

potyvirus tobacco vein mottling virus. *Proceedings of the National Academy of the Sciences, USA.*, **86**, 3509-3513.

DUFFUS, J.E. (1972). Beet western yellows virus. *CMI/AAB Descriptions of Plant Viruses*, no. 89.

DUFFUS, J.E. (1977). Serological relationships among beet western yellows, barley yellow dwarf and soybean dwarf viruses. *Phytopathology*, **67**, 1197-1201.

ESAU, K. & HOEFERT, L.L. (1972). Development of infection with beet western yellows virus in the sugarbeet. *Virology*, **48**, 724-738.

ESKANDARI, F., SYLVESTER, E.S. & RICHARDSON, J. (1979). Evidence for lack of propagation of potato leafroll virus in its aphid vector, *Myzus persicae*. *Phytopathology*, **69**, 45-47.

FALK, B.W., CHIN, L-S. & DUFFUS, J.E. (1989). Complementary DNA cloning and hybridisation analysis of beet western yellows luteovirus RNAs. *Journal of General Virology*, **70**, 1301-1309.

FALK, B.W. & DUFFUS, J.E. (1984). Identification of small single- and double-stranded RNAs associated with severe symptoms in beet western yellows virus-infected *Capsella bursa-pastoris*. *Phytopathology*, **74**, 1224-1229.

FALK, B.W., DUFFUS, J.E. & MAISS, T.J. (1979). Transmission, host range and serological properties of the viruses that cause lettuce speckles disease. *Phytopathology*, **69**, 612-617.

FAUSTMANN, O., KERN, R., SANGER, H.L. & MUHLBACH, M.P. (1986). Potato spindle tuber viroid (PSTV) RNA oligomers of (+) and

(-) polarity are synthesised in potato protoplasts after liposome mediated infection with PSTV. *Virus Research*, **4**, 213-227.

FEINBERG, A.P. & VOGELSTEIN, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry*, **122**, 6-13.

FEINBERG, A.P. & VOGELSTEIN, B. (1984). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry*, **137**, 266-267.

FERARRA, D.M. & TAVANTZIS, S.M. (1986). Expression of extreme resistance to potato virus X (PVX) in *Solanum acaule* protoplasts. *Phytopathology*, **76**, 652.

FRENCH, R. & AHLQUIST, P. (1987). Intercistronic as well as terminal sequences are required for efficient amplification of brome mosaic virus RNA-3. *Journal of Virology*, **61**, 1457-1465.

FRENCH, R. & AHLQUIST, P. (1988). Characterisation and engineering of sequences controlling *in vivo* synthesis of brome mosaic virus subgenomic RNA. *Journal of Virology*, **62**, 2411-2420.

FRENCH, R., JANDA, M. & AHLQUIST, P. (1986). Bacterial gene inserted in an engineered RNA virus: efficient expression in monocotyledonous plant cells. *Science*, **231**, 1294-1297.

FROMM, M., TAYLOR, L.P. & WALBOT, V. (1985). Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proceedings of the National Academy of the Sciences, USA*, **82**, 5824-5828.

FUENTES, A.L. & LEON, P. (1986). Replication of bean rugose mosaic in bean (*Phaseolus vulgaris*) protoplasts from susceptible and resistant strains. *Phytopathology*, **76**, 1096.

FUKUNAGA, K. & FURUSAWA, I. (1981). Infection of turnip protoplasts with radish mosaic virus and its RNA. *Annals of the Phytopathological Society of Japan*, 47, 534-540.

FUKUNAGA, Y., NAGATA, T. & TAKEBE, I. (1981). Liposome mediated infection of plant protoplasts with tobacco mosaic virus RNA. *Virology*, **113**, 752-760.

FURUSAWA, I. & OKUNO, T. (1978). Infection with BMV of mesophyll protoplasts from five plant species. *Journal of General Virology*, **40**, 489- 491.

GARGOURI, R., JOSHI, R.L., BOL, J.F., ASTIER-MANIFACIER, S. & HAENNI, A-L. (1989). Mechanisms of synthesis of turnip yellow mosaic coat protein subgenomic RNA *in vivo*. *Virology*, **171**, 386-393.

GHOSH, A., RUTGERS, T., KE-QIANG, M. & KAESBERG, P. (1981). Characteristics of the coat protein mRNA of southern bean mosaic virus and its relationship to the genomic RNA. *Journal of Virology*, **39**, 87-92.

GILDOW, F.E. (1987). Virus-membrane interactions involved in circulative transmission by aphids. *Current Topics in Vector Research*, **4**, 93-120.

GILDOW, F.E. & ROCHOW, W.F. (1980a). Role of accessory salivary glands in aphid transmission of barley yellow dwarf virus. *Virology*, **104**, 97-108.

GILDOW, F.E. & ROCHOW, W.F. (1980b). Transmission interference between two isolates of barley yellow dwarf virus in *Macrosiphum avenae*. *Phytopathology*, **70**, 122-126.

GILL, C.C. & CHONG, J. (1976). Differences in cellular ultrastructural alterations between variants of barley yellow dwarf virus. *Virology*, **75**, 33 - 47.

GILL, C.C. & CHONG, J. (1979). Cytopathological evidence for the division of barley yellow dwarf virus isolates into two subgroups. *Virology*, **95**, 59-69.

GILL, C.C. & CHONG, J. (1981). Vascular cell alterations and predisposed xylem infection in oats by inoculation with paired barley yellow dwarf viruses. *Virology*, **114**, 405-413.

GOFFINET, D. & VERHOYEN, M. (1979). Infection of tobacco protoplasts by potato virus Y. *Parasitica*, **35**, 25-33.

GOODMAN, R.M. & BIRD, J. (1978). Bean golden mosaic virus. *CMI/AAB Descriptions of Plant Viruses*, no. 192.

GOULDEN, M.G., LOMONOSSOFF, G.P., DAVIES, J.W. & WOOD, K.R. (1990). The complete nucleotide sequence of PEBV RNA-2 reveals the presence of a novel open reading frame and provides insights into the structure of tobraviral subgenomic promoters. *Nucleic Acids Research*, **18**, 4507-4512.

GRONING, B.R., ABOUZID, A. & JESKE, H. (1987). Single-stranded DNA from abutilon mosaic virus is present in the plastids of infected

Abutilon sellovianum. Proceedings of the National Academy of the Sciences, USA., **84**, 8996-9000.

HAMMOND, J., LISTER, R.M. & FOSTER, T.E. (1983). Purification, identity and some properties of an isolate of barley yellow dwarf virus from luteoviruses. *Journal of General Virology*, **64**, 667-676.

HANOLD, D., MISUMI, M. & RANDLES, J.W. (1986). Replication of velvet tobacco mottle virus and Nicotiana velutina mosaic virus in protoplasts. *Biennial Report of the Waite Agricultural Research Institute*, 1984-85, pp150. University of Adelaide, South Australia.

HARRISON, B.D. (1958). Studies on the behaviour of potato leafroll and other viruses in the body of their aphid vector *Myzus persicae* (Sulz.). *Virology*, **6**, 265-277.

HARRISON, B.D. (1984). Potato leafroll virus. *CMI/AAB Descriptions of Plant Viruses*, no. 291.

HARRISON, B.D., KUBO, S., ROBINSON, D.J. & HUTCHESON, A.M. (1976). The multiplication cycle of tobacco rattle virus in tobacco mesophyll protoplasts. *Journal of General Virology*, **33**, 237-248.

HARRISON, B.D. & MAYO, M.A. (1983). The use of protoplasts in plant virus research. In *Use of Tissue Culture and Protoplasts in Plant Pathology*, ch. 4, pp 69-85. Eds. J.P. Helgeson and B.J. Deverall. Academic Press, London.

HARRISON, B.D. & NIXON, H.L. (1960). Purification and electron microscopy of three soil-borne plant viruses. *Virology*, **12**, 104-117.

HENRIQUES, M-I.C. & MORRIS, T.J. (1979). Evidence for different replicative strategies in the plant tombusviruses. *Virology*, **99**, 66-74.

HEWINGS, A.D. & D'ARCY, C.J. (1986). Comparative characterisation of two luteoviruses: beet western yellows virus and barley yellow dwarf virus. *Phytopathology*, **76**, 1270-1274.

HIBI, T., REZELMAN, G. & VAN KAMMEN, A. (1975). Infection of cowpea mesophyll protoplasts with cowpea mosaic virus. *Virology*, **64**, 308-318.

HIRAI, S. & AMEMIYA, Y. (1989). Studies on the resistance of melon cultivars to cucumber mosaic virus (1) Virus multiplication in leaves or mesophyll protoplasts from a susceptible and a resistant cultivar. *Annals of the Phytopathological Society of Japan*, **55**, 458-465.

HOWELL, S.H. & HULL, R. (1978). Replication of cauliflower mosaic virus and transcription of its genome in turnip leaf protoplasts. *Virology*, **86**, 468-481.

HUBER, R., REZELMAN, G., HIBI, T. & VAN KAMMEN, A. (1977). Cowpea mosaic virus infection of protoplasts from Samsun tobacco leaves. *Journal of General Virology*, **34**, 315-323.

HULL, R. & ADAMS, A.N. (1968). Groundnut rosette and its assistor virus. *Annals of Applied Biology*, **62**, 139-145.

HUSSAIN, M.M., MELCHER, H., WHITTLE, T., WILLIAMS, A., BRANNAN, C.M. & MITCHELL, Jr., E.D. (1987). Replication of cauliflower mosaic virus DNA in leaves and suspension culture protoplasts of cotton. *Plant Physiology*, **83**, 633-639.

IMAIZUMI, S. & KUBO, S. (1980). Detection of tobacco necrotic dwarf virus antigen in plant tissues by fluorescent antibody staining. *Annals of the Phytopathological Society of Japan*, **46**, 54-56.

IWAKI, M., ROECHAN, M., HIBINO, H., TOCHIHARA, H. & TANTERA, D.M. (1980). A persistent aphidborne virus of soybean, Indonesian soybean dwarf virus. *Plant Disease*, **64**, 1027-1030.

JARVIS, N.P. & MURAKISHI, H.H. (1980). Infection of protoplasts from soybean cell culture with southern bean mosaic and cowpea mosaic viruses. *Journal of General Virology*, **48**, 365-376.

JENSEN, S.G. (1969). Occurrence of virus particles in the phloem tissue of BYDV-infected barley. *Virology*, **38**, 83-91.

JOERSBO, M. & BRUNSTEDT, J. (1990). Inoculation of sugar beet protoplasts with beet necrotic yellow vein virus particles by mild sonication. *Journal of Virological Methods*, **29**, 63-70.

JOHNSON, J.E. & ARGOS, P. (1985). Virus particle stability and structure. *In The Plant Viruses, Vol. I, Polyhedral Virions with Tripartite Genomes*. pp. 19-56. Ed. R.I.B. Francki. Plenum Press, London.

JONES, R.W. & JACKSON, A.O. (1990). Replication of sonchus yellow net virus in infected protoplasts. *Virology*, **179**, 815-820.

JONES, R.W., JACKSON, A.O. & MONIS, T.J. (1990). Defective-interfering RNAs and elevated temperatures inhibit replication of tomato bushy stunt virus in inoculated protoplasts. *Virology*, **176**, 539-545.

JUNG, J-L., BOUZOUBAA, S. & HAHNE, G. (1991). Visualisation of RNA uptake and expression on the single protoplast level. Unpublished.

KAESBERG, P. (1987). Organisation of tripartite plant virus genomes: the genome of brome mosaic virus. In *The Molecular Biology of the Positive Strand RNA Viruses*, ch. 13, pp 219-236. Eds. D.J. Rowlands, M.A. Mayo & B.W. Mahy. Academic Press, London.

KAGI, T., OSAKI, T. & INOUYE, T. (1975). Broad bean wilt virus infection of broad bean protoplasts. *Annals of the Phytopathological Society of Japan*, **41**, 107-108.

KANO, H. (1985). Effects of light and inhibitors of photosynthesis and respiration on the multiplication of tobacco mosaic virus in tobacco protoplasts. *Plant Cell Physiology*, **26**, 1241-1249.

KASSANIS, B. (1950). Heat inactivation of leaf roll virus in potato tubers. *Annals of Applied Biology*, **37**, 339-341.

KASSANIS, B., WHITE, R.F., TURNER, R.H. & WOODS, R.D. (1977). The mechanism of virus entry during infection of tobacco protoplasts with TMV. *Phytopathologische Zeitschrift*, **88**, 215-228.

KEESE, P., MARTIN, R.R., KAWCHUCK, L.M., WATERHOUSE, P.M. & GERLACH, W.L. (1990). Nucleotide sequences of an Australian and a Canadian isolate of potato leafroll luteovirus and their relationships with two European isolates. *Journal of General Virology*, **71**, 719-724.

KLUGE, S., KIRSTEN, U. & OERTEL, C. (1983). Infection of *Dianthus* protoplasts with carnation mottle virus. *Journal of General Virology*, **64**, 2485-2487.

KOIKE, M., HIBI, T. & YORA, K. (1976). Tobacco mosaic virus infection of cowpea protoplasts. *Annals of the Phytopathological Society of Japan*, **42**, 105.

KOIKE, M., HIBI, T. & YORA, K. (1977). Infection of cowpea mesophyll protoplasts with cucmber mosaic virus. *Virology*, **83**, 413-416.

KOJIMA, M., SHIKATA, E., SUGAWARA, M. & MURAYAMA, D. (1969). Purification and electron microscopy of potato leafroll virus. *Virology*, **39**, 162-174.

KUBO, S. (1981). Tobacco necrotic dwarf virus. *CMI/AAB Descriptions of Plant Viruses*, no. 234.

KUBO, S., HARRISON, B.D. & BARKER, H. (1975a). Defined conditions for growth of tobacco plants as sources of protoplasts for virus infection. *Journal of General Virology*, **28**, 255-257.

KUBO, S., HARRISON, B.D. & ROBINSON, D.J. (1974). Effect of phosphate on the infection of tobacco protoplasts by tobacco rattle virus. *Intervirology*, **3**, 382-387.

KUBO, S., HARRISON, B.D., ROBINSON, D.J. & MAYO, M.A. (1975b). Tobacco rattle virus in tobacco mesophyll protoplasts: infection and virus multiplication. *Journal of General Virology*, **27**, 293-304.

KUBO, S., ROBINSON, D.J., HARRISON, B.D. & HUTCHESON, A.M. (1976). Uptake of tobacco rattle virus by tobacco protoplasts and the effect of phosphate on infection. *Journal of General Virology*, **30**, 287-298.

LOESCH-FRIES, L.S. & HALL, T.C. (1982). *In vivo* aminoacylation of brome mosaic and barley stripe mosaic virus RNAs. *Nature*, **298**, 771-773.

LOMMEL, S.A., KENDALL, T.L., XIONG, Z. & NUTTER, R.C. (1991). Identification of the maize chlorotic mottle virus capsid protein cistron and characterisation of its subgenomic messenger RNA. *Virology*, **181**, 382-385.

MAEKAWA, K., FURUSAWA, I. & OKUNO, T. (1981). Effects of actinomycin D and ultraviolet irradiation on multiplication of brome mosaic virus in host and non-host cells. *Journal of General Virology*, **53**, 353-356.

MARCO, S. (1981). Reducing potato leafroll virus (PLRV) in potato by means of baiting aphids to yellow surfaces and protecting crops by coarse nets. *Potato Research*, **24**, 21-31.

MARSH, L.E., DREHER, T.W. & HALL, T.C. (1988). Mutational analysis of the core and modulator sequences of the BMV RNA 3 subgenomic promoter. *Nucleic Acids Research*, **16**, 981-995.

MARTELLI, G.P. (1991). The nepovirus group. In *Classification and Nomenclature of Viruses. Fifth Report of the International Comittee on Taxonomy of Viruses*, pp. 368-371. Eds. R.I.B. Francki, C.M. Fauquet, D.L. Knudson & F. Brown. Archives of Virology Supplementum 2, Springer-Verlag, Wien.

MARTIN, R.R. & D'ARCY, C.J. (1990). Relationships among luteoviruses based on nucleic acid hybridisation and serological studies. *Intervirology*, **31**, 23-30.

MARTIN, R.R., KEESE, P.K., YOUNG, M.J., WATERHOUSE, P.M. & GERLACH, W.L. (1990). Evolution and molecular biology of luteoviruses. *Annual Review of Phytopathology*, **28**, 341-363.

MASSALSKI, P.R. & HARRISON, B.D. (1987). Properties of monoclonal antibodies to potato leafroll luteovirus and their use to distinguish virus isolates differing in aphid transmissibility. *Journal of General Virology*, **68**, 1813-1821.

MATZEIT, V., SCHAEFER, S., KAMMANN, M., SCHALK, H-J., SCHELL, J. & GRONENBORN, B. (1991). Wheat dwarf virus vectors replicate and express foreign genes in cells of monocotyledonous plants. *The Plant Cell*, **3**, 247-258.

MAULE, A.J. (1983). Infection of protoplasts from several *Brassica* species with cauliflower mosaic virus following inoculation using polyethylene glycol. *Journal of General Virology*, **64**, 2655-2660.

MAULE, A.J., BOULTON, M.I., EDMUNDS, C. & WOOD, K.R. (1980a). Polyethylene glycol-mediated infection of cucumber protoplasts by cucumber mosaic virus and virus RNA. *Journal of General Virology*, **47**, 199 - 203.

MAULE, A.J., BOULTON, M.I. & WOOD, K.R. (1980b). Resistance of cucumber protoplasts to cucumber mosaic virus: a comparative study. *Journal of General Virology*, **51**, 271-279.

MAYO, M.A. & BARKER, H. (1983). Effects of actinomycin D on the infection of tobacco protoplasts by four viruses. *Journal of General Virology*, **64**, 1775-1780.

MAYO, M.A. & BARKER, H. (1984). Translation products and RNA species of potato leafroll virus. *Annual Report of the Scottish Crop Research Institute, 1983*, pp. 186-187.

MAYO, M.A., BARKER, H. & HARRISON, B.D. (1982b). Specificity and properties of the genome-linked proteins of nepoviruses. *Journal of General Virology*, **59**, 149-162.

MAYO, M.A., BARKER, H., ROBINSON, D.J., TAMADA, T. & HARRISON, B.D. (1982a). Evidence that potato leafroll virus RNA is positive stranded, is linked to a small protein and does not contain polyadenylate. *Journal of General Virology*, **59**, 163-167.

MAYO, M.A. & JOLLY, C.A. (1991). The 5'-terminal sequence of potato leafroll virus RNA: evidence of recombination between virus and host RNA. *Journal of General Virology*, **72**, 2591-2595.

MAYO, M.A. & ROBERTS, I.M. (1979). Some effects of buffers on the infectivity and appearance of virus inocula used for tobacco protoplasts. *Journal of General Virology*, **44**, 691-698.

MAYO, M.A. & ROBINSON, D.J. (1977). In *Acides Nucleique et Synthese des Proteines chez les Vegetaux.* Eds. L. Bogorad and J.H. Weil. Colloques Internationaux, no. 261, p. 699. CNRS, Paris.

MAYO, M.A., ROBINSON, D.J. & BARKER, H. (1984). Translation products and RNA species of potato leafroll virus. *Annual Report of the Scottish Crop Research Institute, 1983*, p. 187.

MAYO, M.A., ROBINSON, D.J., JOLLY, C.A. & HYMAN, L. (1989). Nucleotide sequence of potato leafroll luteovirus RNA. *Journal of General Virology*, **70**, 1037-1051.

MILLER, W.A., DREHER, T.W. & HALL, T.C. (1985). Synthesis of brome mosaic virus subgenomic RNA *in vitro* by internal initiation on (-) sense genomic RNA. *Nature*, **313**, 68-70.

MILLER, W.A., HERCUS, T., WATERHOUSE, P.M. & GERLACH, W.L. (1991). A satellite RNA of barley yellow dwarf virus contains a novel hammerhead structure in the self-cleavage domain. *Virology*, **183**, 711-720.

MILLER, W.A., WATERHOUSE, P.M. & GERLACH, W.L. (1988). Sequence and organisation of barley yellow dwarf virus genomic RNA. *Nucleic Acids Research*, **16**, 6097-6111.

MORCH, M-D. & HAENNI, A-L. (1987). Organisation of plant virus genomes that comprise a single RNA molecule. In *The Molecular Biology of the Positive Strand RNA Viruses*, ch. 10, pp. 153-175. Eds.

D.J. Rowlands, M.A. Mayo & B.W.J. Mahy. Academic Press, London.

MORRIS, J. & DE ZOETEN, G.A. (1990). Characterisation and translation studies of potato virus S RNA. *Phytopathology*, **80**, 441-445.

MORRIS-KRSINICH, B.A.M., HULL, R. & RUSSO, M. (1979). Infection of turnip leaf protoplasts with turnip rosette virus. *Journal of General Virology*, **43**, 339-347.

MOTOYOSHI, F., BANCROFT, J.B. & WATTS, J.W. (1974a). The infection of tobacco protoplasts with a variant of brome mosaic virus. *Journal of General Virology*, **25**, 31-36.

MOTOYOSHI, F., BANCROFT, J.B., WATTS, J.W. & BURGESS, J. (1973). The infection of tobacco protoplasts with cowpea chlorotic mottle virus and its RNA. *Journal of General Virology*, **20**, 177-193.

MOTOYOSHI, F. & HULL, R. (1974). The infection of tobacco protoplasts with pea enation mosaic virus. *Journal of General Virology*, **24**, 89-99.

MOTOYOSHI, F., HULL, R. & FLACK, I.H. (1975). Infection of tobacco mesophyll protoplasts by alfalfa mosaic virus. *Journal of General Virology*, **27**, 263-266.

MOTOYOSHI, F. & OSHIMA, N. (1975). Infection with tobacco mosaic virus of leaf mesophyll protoplasts from susceptible and resistant lines of tomato. *Journal of General Virology*, **29**, 81-91.

MOTOYOSHI, F. & OSHIMA, N. (1976). The use of Tris-HCl buffer for inoculation of tomato protoplasts with tobacco mosaic virus. *Journal of General Virology*, **32**, 311-314.

MOTOYOSHI, F. & OSHIMA, N. (1979). Standardisation in inoculation procedure and effect of a resistance gene on infection of tomato protoplasts with tobacco mosaic virus RNA. *Journal of General Virology*, **44**, 801-806.

MOTOYOSHI, F., WATTS, J.W. & BANCROFT, J.B. (1974b). Factors influencing the infection of tobacco protoplasts by cowpea chlorotic mottle virus. *Journal of General Virology*, **25**, 245-256.

MUHLBACH, H-P., CAMACHO-HENRIQUEZ, A. & SANGER, H.L. (1977). Infection of tomato protoplasts by ribonucleic acid of tobacco mosaic virus and by viroids. *Phytopathologische Zeitschrift*, **90**, 289-305.

MUHLBACH, H-P. & SANGER, H.L. (1977). Multiplication of cucumber pale fruit viroid in inoculated tomato leaf protoplasts. *Journal of General Virology*, **35**, 377-386.

MURAKISHI, H.H. (1968). Infection of tomato callus cells in suspension with TMV-RNA. *Phytopathology*, **58**, 993-996.

MURANT, A.F. (1970). Arabis mosaic virus. *CMI/AAB Descriptions of Plant Viruses*, no. 16.

MURPHY, J.F., D'ARCY, C.J. & CLARK, Jr., J.M. (1989). Barley yellow dwarf virus RNA has a 5'-terminal genome-linked protein. *Journal of General Virology*, **70**, 2253-2256.

NATSUAKI, T., MAYO, M.A., JOLLY, C.A. & MURANT, A.F. (1991). Nucleotide sequence of raspberry bushy dwarf virus RNA-2: a bicistronic component of a bipartite genome. *Journal of General Virology*, **72**, 2183-2189.

NELSON, G.A. & TORFASON, W.E. (1974). Associative effects of leaf roll and ring rot on disease expression and yield of potatoes. *American Potato Journal*, **51**, 12-15.

NITTA, N., TAKANAMI, Y., KUWATA, S. & KUBO, S. (1988). Inoculation with RNAs 1 and 2 of cucumber mosaic virus induces viral RNA replicase activity in tobacco mesophyll protoplasts. *Journal of General Virology*, **69**, 2695-2700.

OKADA, K., NAGATA, T. & TAKEBE, I. (1988). Co-electroporation of rice protoplasts with RNA of cucumber mosaic and tobacco mosaic viruses. *Plant Cell Reports*, **7**, 333-336.

OKUNO, T. & FURUSAWA, I. (1978). Factors influencing the infection of barley mesophyll protoplasts with brome mosaic virus RNA. *Journal of General Virology*, **41**, 63-75.

OKUNO, T. & FURUSAWA, I. (1979). RNA polymerase activity and protein synthesis in brome mosaic virus-infected protoplasts. *Virology*, **99**, 218-225.

OKUNO, T., FURUSAWA, I. & HIRUKI, C. (1977). Infection of barley protoplasts with brome mosaic virus. *Phytopathology*, **67**, 610-615.

OSAKI, T. & INOUYE, T. (1981). Tobacco leaf curl virus. *CMI/AAB Descriptions of Plant Viruses*, no. 232.

OSMAN, T.A.M. & BUCK, K.W. (1987). Replication of red clover necrotic mosaic virus RNA in cowpea protoplasts: RNA 1 replicates independently of RNA 2. *Journal of General Virology*, **68**, 289-296.

OTSUKI, Y. & TAKEBE, I. (1969a). Fluorescent antibody staining of tobacco mosaic virus antigen in tobacco mesophyll protoplasts. *Virology*, **38**, 497-499.

OTSUKI, Y. & TAKEBE, I. (1969b). Isolation of intact mesophyll cells and their protoplasts from higher plants. *Plant and Cell Physiology*, **10**, 917-921.

OTSUKI, Y. & TAKEBE, I. (1973). Infection of tobacco mesophyll protoplasts by cucumber mosaic virus. *Virology*, **52**, 433-438.

PAJE-MANALO, L.L. & LOMMEL, S.A. (1989). Independent replication of red clover necrotic mosaic virus RNA-1 in electroporated host and non-host *Nicotiana* species protoplasts. *Phytopathology*, **79**, 457-461.

PALIWAL, Y.C. (1978). Purification and some properties of barley yellow dwarf virus. *Phytopathologische Zeitschrift*, **92**, 240-246.

PALUKAITIS, P. (1984). Detection and characterisation of subgenomic RNA in plant viruses. *Methods in Virology*, vol. **VII**, ch. 9, pp. 259-317.

PAPPU, H.R. & HIRUKI, C. (1988). Replication of sweet clover necrotic mosaic virus in cowpea protoplasts. *Canadian Journal of Plant Pathology*, **10**, 110-115.

PEDEN, K.W.C. & SYMONS, R.H. (1973). Cucumber mosaic virus contains a functionally divided genome. *Virology*, **53**, 487-492.

PETERS, D. (1965). The purification of virus-like particles from the aphid *Myzus persicae*. *Virology*, **26**, 159-161.

PRAKASH, J. & FOXE, M.J. (1985). Optimisation of conditions for infection of isolated potato protoplasts with potato virus X. *Archives of Virology*, **85**, 269-280.

PRILL, B., MAISS, E., KATUL, L. & CASPER, R. (1990). Nucleotide sequence of the bean leafroll luteovirus coat protein gene. *Nucleic Acids Research*, **18**, 5544.

PROLL, E., EISBEIN, K., HAASE, D. & RICHTER, J. (1985). Ein Isolat des Gerstengelbverzwergungs-virus mit einer top-Komponente. *Archiv fur Phytopathologie und Pflanzenschutz*, **21**, 243-245.

RAJESHWARI, R. & MURANT, A.F. (1988). Purification and particle properties of groundnut rosette assistor virus and production of a specific antiserum. *Annals of Applied Virology*, **112**, 403-414.

RANDLES, J.W. (1991). The luteovirus group. In *Classification and Nomenclature of Viruses. Fifth Report of the International Committee on Taxonomy of Viruses*, pp. 309-311. Eds. R.I.B. Francki, C.M. Fauquet, D.L. Knudson & F. Brown. Archives of Virology Supplementum 2, Springer-Verlag, Wien.

RAO, D.V. & HIRUKI, C. (1978). Infection of cowpea mesophyll protoplasts with clover yellow mosaic virus. *Journal of General Virology*, **38**, 303-311.

REGISTER III, J.C. & BEACHY, R.N. (1988). Resistance to TMV in transgenic plants results from interference with an early event in infection. *Virology*, **166**, 524-532.

RENAUDIN, J., BOVE, J.M., OTSUKI, Y. & TAKEBE, I. (1975). Infection of brassica leaf protoplasts by turnip yellow mosaic virus. *Molecular and General Genetics*, **141**, 59-68.

REISTERER, C. & ADAM, G. (1981). Infection of tobacco protoplasts with a plant rhabdovirus. *Abstracts of Fifth International Congress of Virology*, Strasbourg, p. 222.

REUNOVA, G.D., TRUBITSYN, A.G. & REIFMAN, V.G. (1988). Effect of actinomycin D on tobacco mosaic virus (TMV) accumulation in isolated tobacco protoplasts under varying light conditions. *Virology*, **163**, 198-200.

ROBINSON, D.J. (1982). Apparent lack of nucleotide sequence homology between the satellite and genome RNA species of tomato black ring virus. *Journal of General Virology*, **58**, 453-456.

ROCHOW, W.F. (1969). Biological properties of four isolates of barley yellow dwarf virus. *Phytopathology*, **59**, 1580-1589.

ROCHOW, W.F. (1970). Barley yellow dwarf virus. *CMI/AAB Descriptions of Plant Viruses*, no. 32.

ROCHOW, W.F. (1972). The role of mixed infections in the transmission of plant viruses by aphids. *Annual Review of Phytopathology*, **10**, 101-124.

ROCHOW, W.F. (1975). Barley yellow dwarf: dependent virus transmission by *Rhopalosiphum maidis* from mixed infections. *Phytopathology*, **65**, 99-105.

ROCHOW, W.F. (1982). Dependent transmission by aphids of barley yellow dwarf luteoviruses from mixed infections. *Phytopathology*, **72**, 302-305.

ROCHOW, W.F. & CARMICHAEL, L.E. (1979). Specificity among barley yellow dwarf viruses in enzyme immunosorbent assays. *Virology*, **95**, 415-420.

ROLLO, F. & HULL, R. (1982). Liposome-mediated infection of turnip protoplasts with turnip rosette virus and its RNA. *Journal of General Virology*, **60**, 359-363.

RUSSO, M. & GALLITELLI, D. (1985). Infection of cowpea protoplasts with cymbidium ringspot virus. *Journal of General Virology*, **66**, 2033-2037.

SAMAC, D.A., NELSON, S.E. & LOESCH-FRIES, L.S. (1983). Virus protein synthesis in alfalfa mosaic virus infected alfalfa protoplasts. *Virology*, **131**, 455-462.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. (1989). Molecular Cloning - A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, New York.

SANDER, E. & MERTES, G. (1984). Use of protoplasts and separate cells in plant virus research. *Advances in Virus Research*, **29**, 215-262.

SANGER, F., NICKLEN, S. & COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of the Sciences, USA.*, **74**, 5463-5467.

SARKAR, S. (1976). Potato leafroll virus contains a double-stranded DNA. *Virology*, **70**, 265-273.

SARKAR, S., UPADHYA, M.D. & MELCHERS, G. (1974). A highly efficient method of inoculation of tobacco mesophyll protoplasts with ribonucleic acid of tobacco mosaic virus. *Molecular and General Genetics*, **135**, 1-9.

SCALLA, R. & ROCHOW, W.F. (1977). Protein component of two isolates of barley yellow dwarf virus. *Virology*, **78**, 576-580.

SCHOELZ, J.E. & ZAITLIN, M. (1989). Tobacco mosaic virus RNA enters chloroplasts *in vivo*. *Proceedings of the National Academy of the Sciences, USA.*, **86**, 4496-4500.

SHALLA, T.A. & PETERSEN, L.J. (1973). Infection of isolated plant protoplasts with potato virus X. *Phytopathology*, **63**, 1125-1130.

SHANKS, M., TOMENIUS, K., CLAPHAM, D., HUSKISSON, N.S., BARKER, P.J., WILSON, I.G., MAULE, A.J. & LOMONOSOFF, G.P. (1989). Identification and subcellular localisation of a putative cell-to-cell transport protein from red clover mottle virus. *Virology*, **173**, 400-407.

SHEPARDSON, S., ESAU, K. & McCRUM, R. (1980). Ultrastructure of potato leaf phloem infected with potato leafroll virus. *Virology*, **105**, 379-392.

SHEPHERD, R.J., FRANCKI, R.I.B., HIRTH, L., HOLLINGS, M., INOUYE, T., MacLEOD, R., PURCIFULL, D.E., SINHA, R.C., TREMAINE, J.H., VALENTA, V. & WETTER, C. (1976). New groups of plant viruses approved by the International Committee on Taxonomy of Viruses, September 1975. *Intervirology*, **6**, 181-184.

SMITH, K.M. (1945). Transmission by insects of a plant virus complex. *Nature*, **155**, 174.

SMITH, K.M. (1946). The transmission of a plant virus complex by aphids. *Parasitology*, **37**, 131-134.

SMITH, O.P. & HARRIS, K.F. (1990). Potato leafroll virus 3' genome organisation: sequence of the coat protein gene and identification of a viral subgenomic RNA. *Phytopathology*, **80**, 609-614.

SMITH, O.P., HARRIS, K.F., TOLER, R.W. & SUMMERS, M.D. (1988). Molecular cloning of potato leafroll virus complementary DNA. *Phytopathology*, **78**, 1060-1066.

STACE-SMITH, R. (1970). Tobacco ringspot virus. *CMI/AAB Descriptions of Plant Viruses*, no. 17.

STACE-SMITH, R. (1985). Tobacco ringspot virus. *CMI/AAB Descriptions of Plant Viruses*, no. 309.

STEGWEE, D. & PONSEN, M.B. (1958). Multiplication of potato leafroll virus in the aphid *Myzus persicae* (Sulz.). *Entomologia Experimentalis et Applicata*, **1**, 291-300.

SUGIMURA, Y. & USHIYAMA, R. (1975). Cucumber green mottle mosaic virus infection and its bearing on cytological alterations in tobacco mesophyll protoplasts. *Journal of General Virology*, **29**, 93-98.

SYMONS, R.H. (1985). Viral genome structure. In *The Plant Viruses: Polyhedral Virions With Tripartite Genomes*. Volume 1, pp. 57-81. Ed. R.I.B. Francki. Plenum Press, New York.

TACKE, E., PRUFER, D., SALAMINI, F. & ROHDE, W. (1990). Characterisation of a potato leafroll luteovirus subgenomic RNA: differential expression by internal translation initiation and UAG suppression. *Journal of General Virology*, **71**, 2265-2272.

TAKANAMI, Y. & KUBO, S. (1979). Enzyme assisted purification of two phloem-limited plant viruses: tobacco necrotic dwarf and potato leafroll. *Journal of General Virology*, **44**, 153-159.

TAKEBE, I. (1975). The use of protoplasts in plant virology. *Annual Review of Phytopathology*, **13**, 105-125.

TAKEBE, I. (1977). Protoplasts in the study of plant virus replication. In *Comprehensive Virology*, Vol. 11, Ch. 5, pp. 237-283. Eds. H. Fraenkel-Conrat and R.R. Wagner. Plenum Press, New York.

TAKEBE, I. & OTSUKI, Y. (1969). Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. *Proceedings of the National Academy of the Sciences, USA*, **64**, 843-848.

TAKEBE, I., OTSUKI, Y. & AOKI, S. (1968). Isolation of tobacco mesophyll cells in intact and active state. *Plant and Cell Physiology*, **9**, 115-124.

TAMADA, T. & HARRISON, B.D. (1980). Factors affecting the detection of potato leafroll virus in potato foliage by enzyme-linked immunosorbent assay. *Annals of Applied Biology*, **95**, 209-219.

TAMADA, T., HARRISON, B.D. & ROBERTS, I.M. (1984). Variation among British isolates of potato leafroll virus. *Annals of Applied Biology*, **104**, 107-116.

TAMADA, T. & KOJIMA, M. (1977). Soybean dwarf virus. *CMI/AAB Descriptions of Plant Viruses*, no. 179.

TOWNSEND, R., WATTS, J. & STANLEY, J. (1986). Synthesis of viral DNA forms in *Nicotiana plumbaginifolia* protoplasts inoculated with cassava latent virus (CLV); evidence for the independent replication of one component of the CLV genome. *Nucleic Acids Research*, **14**, 1253-1266.

TREMAINE, J.H. & HAMILTON, R.I. (1983). Southern bean mosaic virus. *CMI/AAB Descriptions of Plant Viruses*, no. 274.

VAN BEEK, N.A.M., LOHUIS, D., DIJKSTRA, J. & PETERS, D. (1985). Morphogenesis of sonchus yellow net virus in cowpea protoplasts. *Journal of Ultrastructural Research*, **90**, 294-303.

VAN DER KUYL, A.C., LANGEREIS, K., HOUWING, C.J., JASPARS, E.M.J. & BOL, J.F. (1990). *cis*-acting elements involved in replication of alfalfa mosaic virus RNAs *in vitro*. *Virology*, **176**, 346-354.

VAN DER KUYL, A.C., NEELEMAN, L. & BOL, J.F. (1991). Deletion analysis of *cis*- and *trans*-acting elements involved in replication of alfalfa mosaic virus RNA-3 *in vivo*. *Virology*, **183**, 687-694.

VAN DER WILK, F., HUISMAN, M.J., CORNELISSEN, B.J.C., HUTTINGA, H. & GOLDBACH, R. (1989). Nucleotide sequence and organisation of potato leafroll virus genomic RNA. *FEBS Letters*, **245**, 51-56.

VAN DUN, C.M.P. & BOL, J.F. (1988). Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus. *Virology*, **167**, 649-652.

VAN DUN, C.M.P., BOL, J.F. & VAN VLOTEN-DOTING, L. (1987). Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. *Virology*, **159**, 299-305.

VEIDT, I., BOUZOUBAA, S.E., LEISER, R.M., ZEIGLER-GRAFF, V., GUILLEY, H., RICHARDS, K. & JONARD, G. (1992). Synthesis of full-length transcripts of beet western yellows virus RNA: messenger properties and biological activities in protoplasts. *Virology*, **186**, 192-200.

VEIDT, I., LOT, H., LEISER, M., SCHEIDECKER, D., GUILLEY, H., RICHARDS, K. & JONARD, G. (1988). Nucleotide sequence of beet western yellows virus RNA. *Nucleic Acids Research*, **16**, 9917-9932.

VINCENT, J.R., LISTER, R.M. & LARKINS, B.A. (1991). Nucleotide sequence analysis and genome organisation of the NY-RPV isolate of barley yellow dwarf virus. *Journal of General Virology*, **72**, 2347-2355.

WATERHOUSE, P.M. (1981). Purification, properties and relationships of carrot red leaf virus, and its interaction with carrot mottle virus. PhD Thesis, University of Dundee.

WATERHOUSE, P.M., GERLACH, W.L. & MILLER, W.A. (1986). Serotype-specific and general luteovirus probes from cloned cDNA sequences of barley yellow dwarf virus. *Journal of General Virology*, **67**, 1273-1281.

WATERHOUSE, P.M., GILDOW, F.E. & JOHNSTONE, G.R. (1988). Luteovirus group. *CMI/AAB Descriptions of Plant Viruses,* no. 339.

WATERHOUSE, P.M., MARTIN, R.R. & GERLACH, W.L. (1989). BYDV-PAV virions contain readthrough protein. *Phytopathology*, **79**, 1215.

WATERHOUSE, P.M. & MURANT, A.F. (1982). Carrot red-leaf virus. *CMI/AAB Descriptions of Plant Viruses*, no. 249.

WATERHOUSE, P.M. & MURANT, A.F. (1983). Further evidence on the nature of the dependence of carrot mottle virus on carrot red leaf virus for transmission by aphids. *Annals of Applied Biology*, **103**, 455-464.

WATSON, M., SERJEANT, E.P. & LENNON, E.A. (1964). Carrot motley dwarf and parsnip mottle viruses. *Annals of Applied Biology*, **54**, 153-166.

WATTS, L.E. (1975). The response of various breeding lines of lettuce to beet western yellows virus. *Annals of Applied Biology*, **81**, 393-397.

WATTS, J.W., KING, J.M. & STACEY, N.J. (1987). Inoculation of protoplasts with viruses by electroporation. *Virology*, **157**, 40-46.

WEIDEMANN, V.H.L. (1982). Zur Vermehrung des Kartoffelblattroll-Virus in der Blattlaus *Myzus persicae* (Sulz.). *Zeitschrift fur Angewandte Entomologie*, **94**, 321-330.

WIDHOLM, J.M. (1972). The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology*, **47**, 189-194.

WIERINGA-BRANTS, D.H., TIMMER, F.A. & ROUWELER, M.H.C. (1978). Infection of cowpea mesophyll protopasts by tobacco mosaic virus and tobacco necrosis virus. *Netherlands Journal of Plant Pathology*, **84**, 239-240.

WOODFORD, J.A.T., HARRISON, B.D., AVEYARD, C.S. & GORDON, S.C. (1983). Insecticidal control of aphids and the spread of potato leafroll virus in potato crops in eastern Scotland. *Annals of Applied Biology*, **103**, 117-130.

WOOLSTON, C.J., REYNOLDS, H.V., STACEY, N.J. & MULLINEAUX, P.M. (1989). Replication of wheat dwarf virus DNA in protoplasts and analysis of coat protein mutants in protoplasts and plants. *Nucleic Acids Research*, **17**, 6029-6041.

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The location of the 5′ end of the potato leafroll luteovirus subgenomic coat protein mRNA

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Northern blot analysis of nucleic acid from potato plant tissues and tobacco protoplasts infected with a Scottish isolate of potato leafroll luteovirus (PLRV) detected the 6 kb genomic RNA and one subgenomic RNA species of about 2·7 kb; RNA extracted from virus particles contained only the genomic species. Blotting with small defined probes suggested that the location of the 5′ end of the subgenomic RNA was between 2380 and 2510 nucleotides from the 3′ end of the PLRV genome (between 3370 and 3500 nucleotides from the 5′ end of PLRV Dutch isolate RNA). When RNA extracted from PLRV-infected or mock-inoculated protoplasts was used as the template for primer extension using primers complementary to the sequence at, or upstream of, the initiation codon of the coat protein gene, a single major infection-specific product was detected. A primer complementary to the sequence between 162 and 179 nucleotides upstream of the coat protein AUG yielded a product of 56 nucleotides. Thus, the subgenomic RNA has a leader sequence of 212 nucleotides, is 2505 nucleotides in length and starts at a position equivalent to 3376 nucleotides from the 5′ end of the PLRV-Dutch genome, 11 nucleotides upstream of the termination codon of the putative polymerase gene. The nucleotide sequence immediately downstream of this position closely resembles that of the 5′ end of the PLRV genomic RNA.

Introduction

Potato leafroll virus (PLRV) causes a destructive disease of potato plants, and, like other luteoviruses, is transmitted by aphids in the persistent manner and is limited to the phloem tissue of its host (Harrison, 1984). The virus particles are isometric, 24 nm in diameter and contain a single-stranded, positive-sense genomic RNA of 5·9 kb (Harrison, 1984; Martin *et al.*, 1990).

When infecting cells, a virus may employ several strategies to ensure expression of all the open reading frames (ORFs) in its genome (Morch & Haenni, 1987). Analysis of the nucleotide sequence of the genome of PLRV has identified six ORFs (Mayo *et al.*, 1989; van der Wilk *et al.*, 1989; Keese *et al.*, 1990) which are thought to be expressed by a variety of strategies, including initiation at downstream AUG codons, shifting between reading frames during translation, suppression of termination codons and translation of subgenomic RNA containing downstream ORFs (Mayo *et al.*, 1989; Bahner *et al.*, 1990; Keese *et al.*, 1990).

A PLRV-specific subgenomic RNA has been detected by Northern blotting experiments with RNA from cells infected with PLRV (Mayo *et al.*, 1984). Previous size estimates have been in the region of 2·5 kb (Smith & Harris, 1990), although Tacke *et al.* (1990) obtained a value of 2·3 kb by locating the 5′-terminal nucleotide 40 residues upstream of the coat protein initiation codon. However, our results using a Scottish isolate of PLRV suggest a larger molecular size and a 5′ origin further upstream.

The published sequence for the Scottish PLRV isolate (Mayo *et al.*, 1989) contains a 5′-terminal sequence different from those of other isolates (Keese *et al.*, 1990). Recent work has shown this sequence to be that of a minor fraction in the PLRV (Scottish) RNA (Mayo & Jolly, 1991). For convenience, sequence coordinates used in this paper refer to those in the sequence of the Dutch isolate (van der Wilk *et al.*, 1989).

Methods

Purification of virus particles. Particles of PLRV, Scottish isolate 1 (Tamada *et al.*, 1984) were purified from leaves and stems of the potato cultivar Maris Piper by the method of Harrison (1984). Purified virus particles were stored at −20 °C in 0·02 M-phosphate buffer pH 7·5. In some experiments, potato or *Physalis floridana* plants infected with isolate 11 (Tamada *et al.*, 1984) or isolate V (Massalski & Harrison, 1987) were used.

Protoplast inoculation and culture. Protoplasts were isolated from *Nicotiana tabacum* cv. Xanthi (Kubo *et al.*, 1975) and inoculated using the indirect poly-L-ornithine (PLO) method described by Kubo *et al.* (1975) and Barker & Harrison (1977); inocula contained 0·2 μg/ml

PLRV, 1 μg/ml PLO and 1×10^6 protoplasts. After inoculation, the protoplasts were incubated in continuous light (10000 lux) at 23 to 24 °C as described by Kubo *et al.* (1975).

The proportion of protoplasts infected was determined by staining with fluorescent antibodies (Kubo *et al.*, 1975) and was between 60 and 80% in the experiments described.

RNA extraction. Protoplasts were pelleted by centrifugation at 6000 r.p.m. for 1 min and the pellet was resuspended in 1 to 2 ml 10 mM-Tris–HCl pH 7·6, 50 mM-NaCl, 5 mM-EDTA, 2% SDS, essentially as described by Robinson (1982). After 15 min at 60 °C, the suspension was mixed with an equal volume of water-saturated phenol and *m*-cresol (9:1, v/v) containing 0·1% 8-hydroxyquinoline, and centrifuged at 10000 r.p.m. for 10 min. The aqueous phase was extracted with the phenol mixture again and RNA was precipitated from this by adding 2·5 volumes of ethanol and 0·1 volumes of 3 M-sodium acetate, pH 6·0.

Gel electrophoresis of RNA. RNA (0·5 μg) from PLRV-infected or buffer-inoculated protoplasts was denatured with formamide, heated at 65 °C and separated by electrophoresis in a 1·2% agarose gel containing formaldehyde, as described by Sambrook *et al.* (1989).

RNA from particles of tobacco mosaic virus (6·4 kb; Goelet *et al.*, 1982) and brome mosaic virus (BMV, 3·2 kb, 2·9 kb, 2·1 kb and 0·9 kb; Symons, 1985) was used as an M_r marker.

Northern blot analysis of RNA. PLRV-specific hybridization probes (Fig. 1) were prepared by three methods.

(i) Excision of cDNA complementary to PLRV RNA from a recombinant plasmid. This DNA, probes A and B, corresponded to nucleotides 5586 to 5882 and 3395 to 3645, respectively (Fig. 1).

(ii) cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Pharmacia) primed on virus template RNA with an 18-mer oligonucleotide complementary to nucleotides 3589 to 3607. Part of the resulting cDNA was amplified by the polymerase chain reaction (Natsuaki *et al.*, 1991) using primers equivalent to positions 3365 to 3391 or complementary to positions 3521 to 3538. The reaction consisted of 30 cycles at 95 °C for 90 s, 55 °C for 90 s, 72 °C for 150 s and 72 °C for 5 min. The DNA (probe C, Fig. 1) was recovered by elution from 1·2% agarose gels.

(iii) DNA was extracted from recombinant M13 phage containing the PLRV sequence between nucleotides 3165 and 3375 (probe D) (Fig. 1) as described by Sambrook *et al.* (1989).

Probes A, B and C were labelled according to the method of Feinberg & Vogelstein (1984). Probe D was labelled using a downstream primer as described by Barker *et al.* (1985), excised by restriction digestion with *Eco*RI and recovered by elution from a 17% acrylamide gel.

Prehybridization and hybridization were as described by Sambrook *et al.* (1989); blots were washed four times with 2 × SSC (0·3 M-NaCl, 0·03 M-sodium citrate), 0·1% SDS and four times with 1 × SSC, 0·1% SDS at 65 °C.

Primer extension. The oligonucleotide primers 5′ AACCACGACCGTACTCAT 3′ (complementary to nucleotides 3588 to 3605, primer 1; Fig. 1) and 5′ TTGTTAACTCGTGTATGCTTGGC 3′ (complementary to nucleotides 3409 to 3426 with five extra nucleotides added at the 5′ end, primer 2; Fig. 1) were phosphorylated using polynucleotide kinase and γ-^{32}P-labelled ATP (370 MBq/ml; Amersham) as described by Sambrook *et al.* (1989).

Samples contained 5 μg RNA from PLRV-infected or buffer-inoculated protoplasts, or 2 μg RNA from purified virus particles. RNA was added to 8 μl H_2O containing 50 ng 5′ ^{32}P-labelled primer and heated at 90 °C for 2 min. Samples were mixed with 2 μl of 80 mM-Tris–HCl pH 8·3, 0·27 M-KCl, 20 mM-DTT, 40 mM-$MgCl_2$ immediately, and placed at 50 °C for 20 min and then at room temperature for 15 min. Reverse transcriptase buffer (86 μl; 20 mM-Tris–HCl pH 8·3, 67·5 mM-KCl, 10 mM-$MgCl_2$, 5 mM-DTT, 1 mM-dATP, 1 mM-dCTP, 1 mM-dGTP, 1 mM-TTP) and 4 μl (15 units) of avian myeloblastosis virus reverse transcriptase (Pharmacia) were added. The mixture was incubated for 90 min at 42 °C and extracted successively with equal volumes of phenol/chloroform (1:1) and chloroform/isoamyl alcohol (25:1). Nucleic acid was recovered by precipitation from 70% ethanol for 30 min at −70 °C and centrifugation for 10 min at 10000 r.p.m.

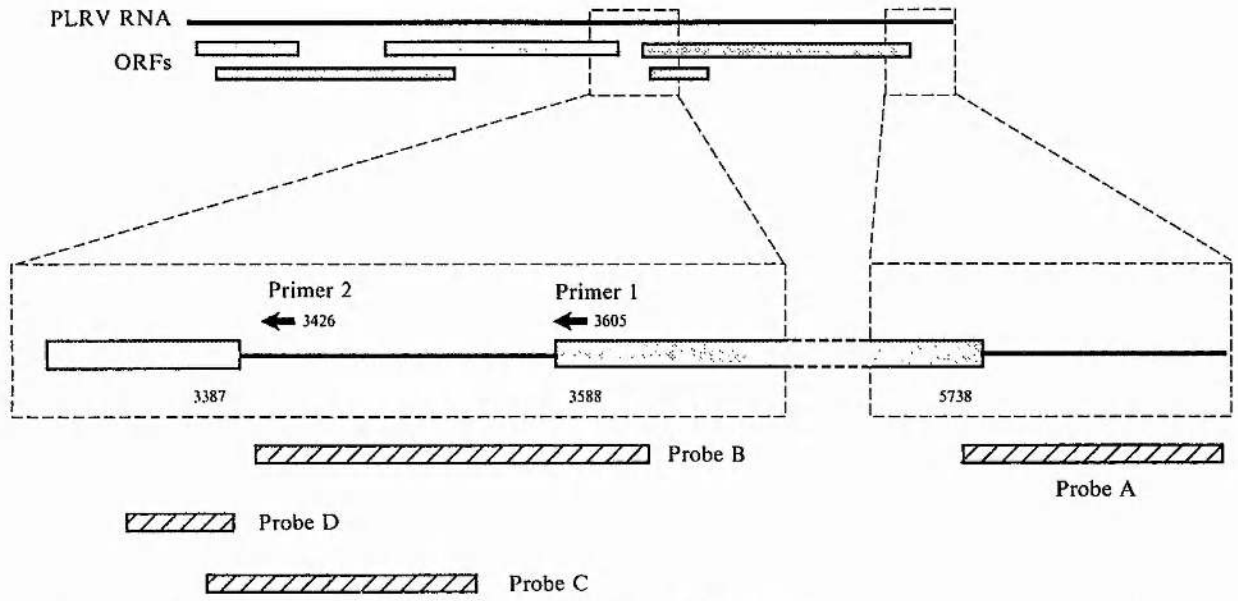

Fig. 1. The location of probes and primers within the sequence of PLRV RNA. The upper diagram represents the entire genome of PLRV, the lower diagram nucleotides 3300 to 3750 and the 3′-terminal 200 nucleotides. The dashed lines indicate the approximate location of these regions in the PLRV RNA sequence. Shaded boxes indicate ORFs, hatched boxes indicate oligonucleotide probes and arrows indicate the location of primers used in primer extension experiments. Numbers indicate the nucleotide positions complementary to the 5′ ends of the primers (arrows) or the edges of ORFs.

Dried nucleic acid was redissolved in 50 μl of 0·3 M-NaOH, incubated at 65 °C for 30 min, mixed with 60 μl of 1 M-Tris–HCl pH 7·5 and ethanol-precipitated at −20 °C overnight.

Products of the primer extension were analysed by electrophoresis at approximately 1·8 kV in an 8% acrylamide gel containing 7 M-urea. The products of sequencing M13mp18 DNA by using the dideoxynucleotide chain-termination technique (Sanger *et al.*, 1977) were allowed to comigrate with the extension products to allow their size to be determined.

Results

Size of subgenomic RNA

Hybridization with probes A, B and C detected two RNA species in RNA from infected protoplasts (Fig. 2, lanes 1, 3 and 5), but only one in RNA from purified virus particles (Fig. 2, lane 2). The size of the RNA from virus particles is 6 kb and was therefore presumed to be the genomic RNA. The faster migrating species detected in infected protoplasts was thus the non-encapsidated subgenomic RNA reported by Mayo *et al.* (1984). The size of the subgenomic RNA was estimated to be 2·7 kb by using each probe in Northern blot analysis. Similar Northern blot analysis of extracts of potato plants infected with PLRV isolates 1, 11 and V, and of *P. floridana* infected with isolate 11 also detected a 2·7 kb subgenomic RNA. Other faint bands were detected in a few analyses; these corresponded in position to rRNA.

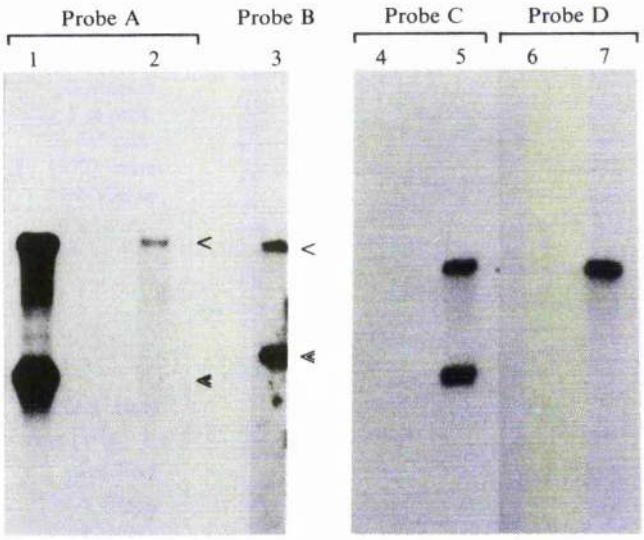

Fig. 2. Northern blot analysis of PLRV RNA. Blots were made in four separate experiments and exposed to probes A to D, respectively. Samples were RNA from PLRV-infected protoplasts (lanes 1, 3, 5 and 7), PLRV particles (lane 2) or buffer-inoculated protoplasts (lanes 4 and 6). Single arrowheads indicate genomic RNA, double arrowheads subgenomic RNA.

Location of viral subgenomic RNA

Probe A detected both RNA species, so the subgenomic RNA must terminate within about 100 nucleotides of the 3′ end; a 2·7 kb molecule terminating at this position would have a 5′ end at about nucleotide 3200. However, when probe D was used in a Northern blot analysis, the subgenomic RNA was not detected (Fig. 2, lane 7). Probe D finishes at nucleotide 3375 and the subgenomic RNA must begin close or 3′ of this point.

When probe C was used, both genomic and subgenomic RNAs were detected (Fig. 2, lane 5). This suggested a more precise location for the 5′ end of the subgenomic RNA. Probe C extends from position 3365 to 3538, so its 3′ end is 51 bases upstream of the start of the coat protein gene [and 11 bases upstream of the position proposed by Tacke *et al.* (1990) for the 5′ end of the subgenomic RNA]. These results suggest that the 5′ end of the subgenomic RNA is between nucleotides 3370 and 3500.

Mapping of the 5′ terminus of the subgenomic RNA by primer extension

The first primer extension experiments were done using primer 1 (Fig. 1), which is complementary to 18 nucleotides at the 5′ terminus of the coat protein gene. Products primed on RNA from infected protoplasts produced several bands, but none was produced when RNA from healthy protoplasts was used (Fig. 3*a*).

Although virus particles do not contain subgenomic RNA, extension from primers annealed to RNA extracted from PLRV particles yielded several bands. These bands were also detected in analyses of samples primed on RNA from PLRV-infected protoplasts and are presumably products formed by synthesis stopping at positions of strong secondary structure. One prominent band of approximately 205 nucleotides was consistently present in extension products primed on RNA from infected protoplasts, but was not found in those primed on virus particle RNA.

This result suggested that the end of the subgenomic RNA was located more than 200 nucleotides upstream of the initiation codon of the coat protein gene. Primer extensions of such a length are known to result in stops (Sambrook *et al.*, 1989) and therefore a second primer, complementary to the sequence 179 nucleotides upstream of the coat protein AUG (primer 2), was used. Extension from this primer yielded a product that comigrated with a dideoxynucleotide-terminated product of 56 nucleotides (Fig. 3*b*). We conclude that the subgenomic RNA starts at nucleotide 3376 (nucleotide 3481 in the sequence of Mayo *et al.*, 1989).

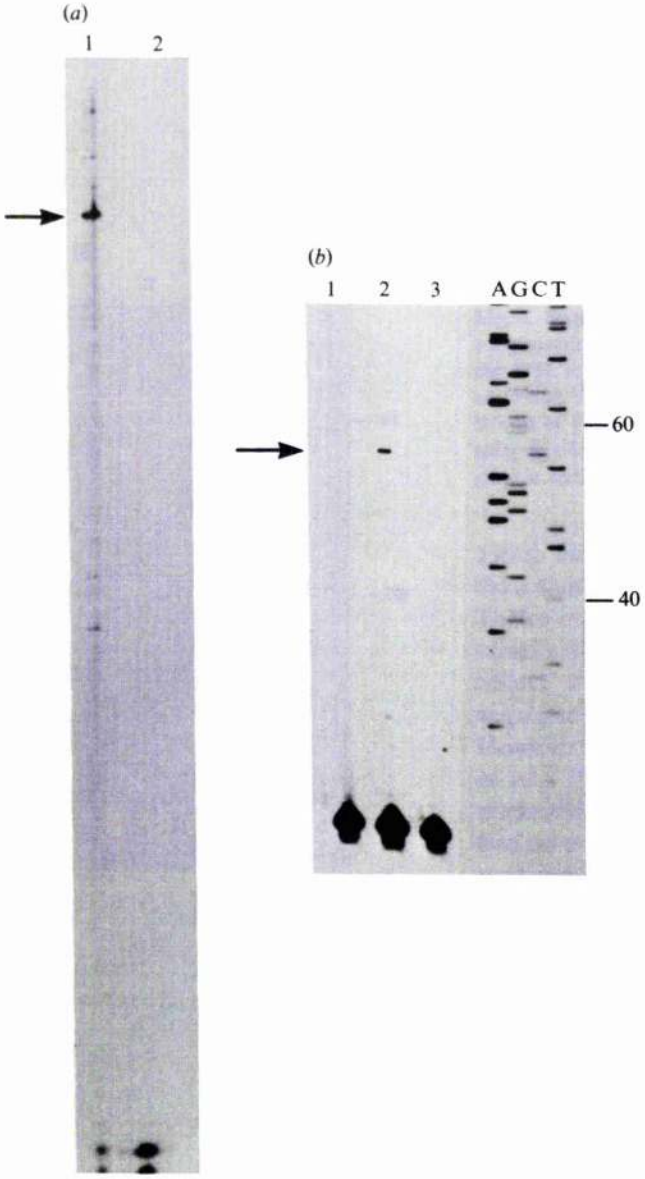

Fig. 3. Primer extension mapping of the 5′ terminus of the subgenomic RNA. (*a*) Extension from primer 1. Samples were RNA from infected protoplasts (lane 1) or mock-inoculated protoplasts (lane 2). The arrow indicates the most prominent infection-specific product. (*b*) Extension from primer 2. Samples were RNA from mock-inoculated protoplasts (lane 1), infected protoplasts (lane 2) or RNA from purified virus particles (lane 3). The arrow indicates the most prominent infection-specific product. Lanes A, G, C and T indicate products of dideoxynucleotide sequencing of M13mp18 DNA. Numbers on the right are the sizes in nucleotides of the oligonucleotide bands indicated.

Discussion

The 5′ terminus of the subgenomic RNA of the Scottish isolate of PLRV has been determined to be nucleotide 3376. The subgenomic RNA is thus 2505 nucleotides in length, which is slightly less than estimates obtained by gel electrophoresis (Martin *et al.*, 1990; Tacke *et al.*, 1990). However, this estimate is 172 nucleotides longer than that obtained for the German isolate of PLRV by Tacke *et al.* (1990) (i.e. 2334 nucleotides with the 5′ terminus at nucleotide 3548). This discrepancy may reflect differences between either the length or the sequence of the subgenomic RNA of the two isolates. However, the published sequences of the isolates (Mayo *et al.*, 1989; Tacke *et al.*, 1989) differ at only five nucleotides in the intergenic region. Moreover, we could find no evidence of a stop 40 nucleotides upstream of the coat protein initiation codon.

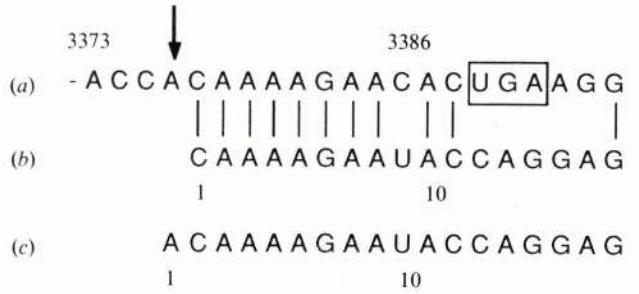

Fig. 4. Sequence similarity between the 5′ termini of the genomic and subgenomic RNAs of PLRV. (*a*, *b*) Sequences from Dutch PLRV (van der Wilk *et al.*, 1989), (*c*) a sequence from Australian PLRV (Keese *et al.*, 1990). The numbers indicate the nucleotide position relative to the 5′ end of the RNA. The arrow indicates the first nucleotide of the subgenomic RNA. The termination codon of the putative polymerase gene is indicated by a box.

The genomic RNA of PLRV contains a direct repeat of the sequence of the first eight (van der Wilk *et al.*, 1989) or nine (Keese *et al.*, 1990) nucleotides in the sequence from nucleotides 3377 or 3376 respectively (Fig. 4). The fact that the sequence of the 5′ ends of the genomic and subgenomic RNAs correspond reinforces the suggestion that the deduced 5′ end of the subgenomic RNA of PLRV is correct. Furthermore, there is a similar correspondence between the 5′-terminal sequence of the genome and a sequence near the 3′ end of the putative polymerase gene in the RNA of beet western yellows virus (BWYV; Veidt *et al.*, 1988). Recent results (V. Ziegler-Graf, personal communication) indicate that this region is where the subgenomic RNA of BWYV starts.

Identical sequences at the 5′ termini of genomic and subgenomic RNAs have also been observed for several other plant viruses. For example, there is a match of 12 nucleotides in maize chlorotic mottle virus RNA (Lommel *et al.*, 1991), up to 10 in tobacco rattle virus RNA (TRV; Cornelissen *et al.*, 1986) and up to 11 in alfalfa mosaic virus RNA (Symons, 1985). As suggested by Cornelissen *et al.* (1986) for TRV RNA, this sequence similarity at the 5′ ends may reflect a replicase recognition signal in the corresponding minus-strand RNA.

Consensus sequences postulated to form promoters for subgenomic RNAs of some plant viruses (although not luteoviruses) have been published (Marsh *et al.*, 1988; Goulden *et al.*, 1990). It is proposed that a core sequence occurs immediately upstream of the subgenomic RNA initiation site of BMV, and that this comprises some 20 bases and has regions of homology with the intercistronic sequences of other plant viruses (French & Ahlquist, 1987, 1988). Some of these features have been found in the intergenic non-coding region of PLRV RNA, in particular a U_nA sequence followed by AAGA (Mayo *et al.*, 1989). However, because it now appears that the subgenomic RNA initiation site is upstream of the intercistronic region, these features are actually downstream of the initiation point. Interestingly, Marsh *et al.* (1988) have noted that at least part of the proposed subgenomic RNA promoter in BMV RNA 3 is downstream of the initiation site of the subgenomic species.

The 5′-terminal sequence of the subgenomic (and genomic) RNA of PLRV is almost identical to that of BWYV (Keese *et al.*, 1990) and there are very similar sequences about 3473 nucleotides from the 5′ ends of the genomic RNA of both viruses (Mayo *et al.*, 1989). However, there are few substantial similarities in sequences thought to be upstream of the 5′ end of subgenomic RNAs of the two viruses. If the mechanism of generation of PLRV subgenomic RNA involves internal initiation on minus-strand templates as has been shown for BMV (Marsh *et al.*, 1988), then perhaps any upstream promoter sequences are specific to each virus whereas the factors recognizing downstream promoter sequences may be less specific. However, this is not so for BMV RNA (Marsh *et al.*, 1988) in which the putative promoter sequences shared with other viruses are upstream rather than downstream of the start of the subgenomic RNA.

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References

BAHNER, I., LAMB, J., MAYO, M. A. & HAY, R. T. (1990). Expression of the genome of potato leafroll virus: readthrough of the coat protein termination codon *in vivo*. *Journal of General Virology* **71**, 2251–2256.

BARKER, H. & HARRISON, B. D. (1977). Infection of tobacco mesophyll protoplasts with raspberry ringspot virus alone and together with tobacco rattle virus. *Journal of General Virology* **35**, 125–133.

BARKER, J. M., MCINNES, J. L., MURPHY, P. J. & SYMONS, R. H. (1985). Dot-blot procedure with ^{32}P DNA probes for the sensitive detection of avocado sunblotch and other viroids in plants. *Journal of Virological Methods* **10**, 87–98.

CORNELISSEN, B. J. C., LINTHORST, H. J. M., BREDERODE, F. TH. & BOL, J. F. (1986). Analysis of the genome structure of tobacco rattle virus strain PSG. *Nucleic Acids Research* **14**, 2157–2169.

FEINBERG, A. P. & VOGELSTEIN, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **137**, 266–267.

FRENCH, R. & AHLQUIST, P. (1987). Intercistronic as well as terminal sequences are required for efficient amplification of brome mosaic virus RNA3. *Journal of Virology* **61**, 1457–1465.

FRENCH, R. & AHLQUIST, P. (1988). Characterization and engineering of sequences controlling *in vivo* synthesis of brome mosaic virus subgenomic RNA. *Journal of Virology* **62**, 2411–2420.

GOELET, P., LOMONOSSOFF, G. P., BUTLER, P. J. G., AKAM, M. E., GAIT, M. J. & KARN, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proceedings of the National Academy of Sciences, U.S.A.* **79**, 5818–5822.

GOULDEN, M. G., LOMONOSSOFF, G. P., DAVIES, J. W. & WOOD, K. R. (1990). The complete nucleotide sequence of PEBV RNA2 reveals the presence of a novel open reading frame and provides insight into the structure of tobraviral subgenomic promoters. *Nucleic Acids Research* **18**, 4507–4512.

HARRISON, B. D. (1984). Potato leafroll virus. *CMI/AAB Descriptions of Plant Viruses*, no. 291.

KEESE, P., MARTIN, R. R., KAWCHUK, L. M., WATERHOUSE, P. M. & GERLACH, W. L. (1990). Nucleotide sequences of an Australian and a Canadian isolate of potato leafroll luteovirus and their relationships with two European isolates. *Journal of General Virology* **71**, 719–724.

KUBO, S., HARRISON, B. D., ROBINSON, D. J. & MAYO, M. A. (1975). Tobacco rattle virus in tobacco mesophyll protoplasts: infection and virus multiplication. *Journal of General Virology* **27**, 293–304.

LOMMEL, S. A., KENDALL, T. L., XIANG, Z. & NUTTER, R. C. (1991). Identification of the maize chlorotic mottle virus capsid protein cistron and characterization of its subgenomic messenger RNA. *Virology* **181**, 382–385.

MARSH, L. E., DREHER, T. W. & HALL, T. C. (1988). Mutational analysis of the core and modulator sequences of the BMV RNA 3 subgenomic promoter. *Nucleic Acids Research* **16**, 981–995.

MARTIN, R. R., KEESE, P. K., YOUNG, M. J., WATERHOUSE, P. M. & GERLACH, W. L. (1990). Evolution and molecular biology of luteoviruses. *Annual Review of Phytopathology* **28**, 341–363.

MASSALSKI, P. R. & HARRISON, B. D. (1987). Properties of monoclonal antibodies to potato leafroll luteovirus and their use to distinguish virus isolates differing in aphid transmissibility. *Journal of General Virology* **68**, 1813–1821.

MAYO, M. A. & JOLLY, C. A. (1991). The 5′-terminal sequence of potato leafroll virus RNA: evidence of recombination between virus and host RNA. *Journal of General Virology* **72**, 2591–2595.

MAYO, M. A., ROBINSON, D. J. & BARKER, H. (1984). *Annual Report of the Scottish Crop Research Institute for 1983*, p. 187.

MAYO, M. A., ROBINSON, D. J., JOLLY, C. A. & HYMAN, L. (1989). Nucleotide sequence of potato leafroll luteovirus RNA. *Journal of General Virology* **70**, 1037–1051.

MORCH, M.-D. & HAENNI, A.-L. (1987). Organisation of plant virus genomes that comprise a single RNA molecule. In *The Molecular Biology of the Positive Strand RNA Viruses*, pp. 153–175. Edited by D. J. Rowlands, M. A. Mayo & B. W. J. Mahy. London: Academic Press.

NATSUAKI, T., MAYO, M. A., JOLLY, C. A. & MURANT, A. F. (1991). Nucleotide sequence of raspberry bushy dwarf virus RNA-2: a bicistronic component of a bipartite genome. *Journal of General Virology* **72**, 2183–2189.

ROBINSON, D. J. (1982). Apparent lack of nucleotide sequence homology between the satellite and genome RNA species of tomato black ring virus. *Journal of General Virology* **58**, 453–456.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, U.S.A.* **74**, 5463–5467.

SMITH, O. P. & HARRIS, K. F. (1990). Potato leafroll virus 3′ genome organization: sequence of the coat protein gene and identification of a viral subgenomic RNA. *Phytopathology* **80**, 609–614.

SYMONS, R. H. (1985). Viral genome structure. In *The Plant Viruses*, vol. 1: *Polyhedral Virions with Tripartite Genomes*, pp. 57–81. Edited by R. I. B. Francki. New York: Plenum Press.

TACKE, E., SARKAR, S., SALAMINI, F. & ROHDE, W. (1989). Cloning of the gene for the capsid protein of potato leafroll virus. *Archives of Virology* **105**, 153–163.

TACKE, E., PRÜFER, D., SALAMINI, F. & ROHDE, W. (1990). Characterization of a potato leafroll luteovirus subgenomic RNA: differential expression by internal translation initiation and UAG suppression. *Journal of General Virology* **71**, 2265–2272.

TAMADA, T., HARRISON, B. D. & ROBERTS, I. M. (1984). Variation among British isolates of potato leafroll virus. *Annals of Applied Biology* **104**, 107–116.

VAN DER WILK, F., HUISMAN, M. J., CORNELISSEN, B. J. C., HUTTINGA, H. & GOLDBACH, R. (1989). Nucleotide sequence and organisation of potato leafroll virus genomic RNA. *FEBS Letters* **245**, 51–56.

VEIDT, I., LOT, H., LEISER, M., SCHEIDECKER, D., GUILLEY, H., RICHARDS, K. & JONARD, G. (1988). Nucleotide sequence of beet western yellows virus RNA. *Nucleic Acids Research* **16**, 9917–9932.

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